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SUPPLEMENTUM

The Fibiger Laboratory Kongens Lyngby Denmark

THE USE OF N DEACETYL-N METHYLCOLCHICINE (COLCEMID) FOR DETERMINATION OF THE REPLICATION TIME OF NON MALIGNANT AND MALIGNANT CELLS CULTIVATED IN VITRO

By

W SAWICKI and J KIELER

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A number of methods for the determination of the duration of the mitotic cycle have been described (*Eigsti & Dustin* 1957 *Painter & Drew* 1959 *Parker* 1961 a o) Some of them are only of historical value but others are valuable and useful methods in common use Autoradiographic methods with the use of labelled thymidine and methods based on cell counts for several consecutive days are most frequently used However all these procedures are time consuming and sometimes complicated This problem has recently been discussed in detail by *Sisken* (1964)

The experiments to be described in the present paper represent an attempt to apply the colcemid method to the determination of the replication time (RT)[1] of non malignant and malignant mouse cells in vitro In order to compare this method with other methods autoradiography with tritiated thymidine as well as daily cell counts for five consecutive days were carried out at the same time

MATERIAL AND METHODS

Six non malignant or malignant murine cells lines were studied in the present work These cell lines will be described in greater detail elsewhere Table 1 shows their origin the time and place of their explantation the culture age at the time of the present experiments and tumour producing capacity in new born hosts The cells were grown on coverslips placed in a modified type of Carrel flasks—the so called Fibiger flasks—with two coverslips in each The medium contained 20 per cent foetal bovine serum and 80 per cent of Eagle s minimum essential medium (*Eagle* 1959) with a two fold increase of essential amino acids a four fold increase of

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[1] In the present paper the term replication t[illegible] without any distinction between doubling time and generation time if not th[illegible] indicated

TABLE 1
Material

Cell line	Origin	Time and place of explantation	Age of cultures at time of investigation	Tumour producing capacity in newborn C_3H mice		
				Total no of mice inoculated	No of takes	Metastases
C_3H 1	19 days old C_3H mouse embryos	1964 Fibiger Laboratory	8 months	42	6	0
C_3H M	Spleen of normal adult C_3H mouse	1964 Fibiger Laboratory	7 months	25	0	0
C_3H L	Lung of same normal adult C_3H mouse	1964 Fibiger Laboratory	7 months	25	25	+
L 929	Subcutis of normal adult C_3H mouse	1940 National Institute of Health Bethesda (*Earle*)	25 years	54	30	+
ELD	Ehrlich's *hyperdiploid* mouse ascites carcinoma	1962 Radiumhemmet Stockholm (*Holmberg*)	4 years	36	35	+
ELT	Ehrlich's *hypertetraploid* mouse ascites carcinoma	1964 Fibiger Laboratory	2 years	[illegible]	22	+

1940–41 treated *in vitro* for 111 days with *methylcholantren* after which the cells were found to be tumour producing on reinoculation into adult C_3H mice (*Earle* & *Nettleship* 1943)

vitamins and glutamine and with glucose at a concentration of 7 mM The gas phase contained 5 per cent CO

Determination of Replication Time (RT) by the Colcemid Method

Twenty four hour old exponentially growing subcultures containing 5 ml of medium each received 0 1 ml of an aqueous solution of N deacetyl N methylcolchicine (Colcemid – Ciba Pharmaceuticals Inc Basel Switzerland) yielding a final concentration of 0 1 μg per ml of medium In some experiments the effect of 0 05 and 1 0 μg was also tested The control cultures received 0 1 ml of water

Coverslips with cells were removed from the flasks at the beginning of the experiment and 1 2 3 4 6 12 18 and 24 hours after the addition of colcemid The cells were washed with a balanced salt solution (BSS) and subjected to an osmotic shock by dipping the coverslips into tap water for a few seconds A flattening of the cells and a slight spread of the chromosomes were thereby obtained which made the identification of various mitotic phases easier After gentle drying with filter paper the cells were fixed in modified Carnoy s fluid consisting of glacial acetic acid and 99 per cent ethanol (1 3) for 30 minutes and stored to the end of the experiment in 70 per cent ethanol Then all the cells were stained with Harris haematoxylin and eosin The mitotic coefficients were finally determined by counting a total of 5 000–7 000 cells from each period of colcemid treatment

Determination of Replication Time by Autoradiography

Pulse labelling experiments were carried out as previously described (*Sawicki et al* 1967) Cells grown on coverslips placed in Fibiger flasks were treated with thymidine 6 ³H (Radiochemical Centre Amersham Bucks England) for 30 minutes Each flask containing two coverslips and 6 ml of medium received 1 × 10 ³ μmoles of tritiated thymidine dissolved in 0 1 ml of distilled water yielding a final concentration of 0 166 μM and an activity of 5 5 × 10 μC per ml Control cultures received 0 1 ml distilled water containing 1 × 10 ³ μmoles of unlabelled thymidine After incubation with thymidine the medium were re fed with the standard medium containing unlabelled thymidine at a concentration of 25 × 10 μM

After re feeding the cultures were incubated for 24–48 hours During this time cover lips from three cultures selected at random were removed for autoradiograph at regular intervals Autoradiography was carried out with the Kodak NTB 2 liquid emulsion as described by *Leblond et al* (1963) and by *Sawicki & Pawinska* (1965) After exposure for 3–5 days the autoradiographs were stained with Harris haematoxylin and eosin All autoradiographs were examined microscopically and in each of them 50–100 labelled and unlabelled metaphases were counted The percentage of labelled metaphases was plotted against time of reincubation after pulse labelling The replication time was calculated from these curves as described by *Painter & Drew* (1959)

Determination of Replication Time by Cell Counts

Cultures used for the determination of replication time by this method were incubated for 5 days Each day ten cultures were selected at random for counting while the medium and the gas phase were renewed in the cultures that remained for further incubation The cells were harvested by trypsinization suspended in BSS and counted in a Bürker Turk haemo tometer The results were read from the growth curves and analysed statistically by the method described by *Dean & Dixon* (1951)

RESULTS

The mitotic indices of colcemid treated cultures are shown in Table 2 As it can be seen the control cultures showed relatively high values being in the range of ca 3–5 per cent In all colcemid treated cultures the ana telo phases and reconstruction phases disappeared already during the first hour of colcemid treatment Simultaneously arrested metaphases and ski anaphases (Fig 1) started to appear

TABLE 2

Mitotic Index and Replication Time (RT) of Six in Vitro Propagated Murine Cell Lines Treated with Colcemid for 1–6 Hours

Cell line	Control	Duration of colcemid treatment (hours) 1		2		3		4		6	
		Mitotic index (MI – per cent) and replication time (RT – hours)§									
		MI	RT	MI	RT	MI	RT	MI	RT	MI	RT
C_3H F	4 5	6 9	14 7	7 9	25 2	10 9	27 5	13 9	27 4	27 4	2[illegible] 0
C_3H M	3 1	2 7	34 1	7 2	27 7	10 3	29 3	13 7	29 2	16 5	3[illegible] 2
C_3H L	4 2	3 8	24 4	6 4	31 3	9 9	30 3	13 3	30 0	16 3	3[illegible] 8
L-929	3 7	4 9	20 4	7 2	27 7	10 2	29 4	14 4	[illegible]7 4	17 8	33 1
ELD	4 0	4 1	24 4	8 1	24 7	10 4	29 4	17 0	23 5	19 9	30 2
ELT	4 6	3 9	26 0	2 4	83	1 6	187	2 0	[illegible]00	2 5	240

Mean values based on counts of a total of 5000–7000 cells

§ Mean values calculated from the corresponding mitotic indices according to the formula $RT \pm \frac{100\ t}{C_t}$ where t is the time of incubation and C_t the mitotic index

With the time of incubation the number of arrested metaphases increased in the colcemid treated cultures in five of the six cell lines studied. Only the ELT cultures did not show any accumulation of metaphases. Figs 2–6 show the accumulation of mitotic figures in the other five cell lines during 8 hours of colcemid treatment. It appears from Fig 2 that a linear accumulation of mitotic figures occurred in



Fig 1

"Ski anaphase" (Levan 1959) in colcemid treated C_3H E culture

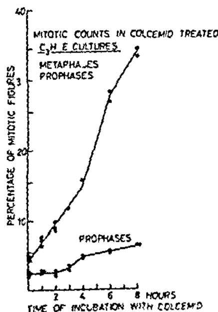


Fig 2

Mitotic counts in colcemid treated C_3H E cultures

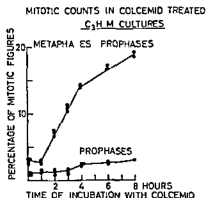


Fig 3
Mitotic counts in colcemid treated C_3H M cultures

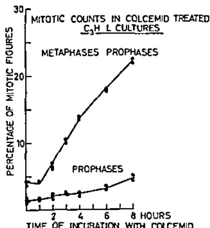


Fig 4
Mitotic counts in colcemid treated C_3H L cultures

the C_3H E cultures from the beginning of the experiment and lasting until the fourth hour of incubation A similar accumulation was seen in the C_3H M C_3H L L-929 and ELD cultures but only after a lag phase of 1 hour (Figs 3–6) The linear curve obtained between the second and the fourth hour of incubation extrapolated back to a point very close to or identical with the zero point

After the fourth hour a further increase in the rate of accumulation of mitotic figures was seen in the C_3H E cultures while the curves for the other four cell lines started to fall off Fig 7 shows the accumula

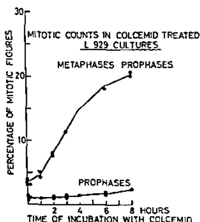


Fig 5
Mitotic counts in colcemid treated L-929 cultures

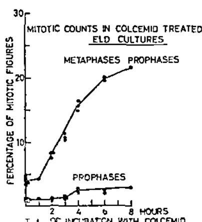


Fig 6
Mitot ounts in olcemid treate ELD ultures

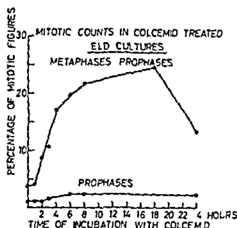


Fig 7
Mitotic counts in colcemid treated FLD cultures

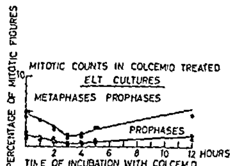


Fig 8
Mitotic counts in colcemid treated ELT cultures

tion of mitotic cells in ELD cultures during 24 hours of colcemid treatment As it is seen the curve continued to fall off after the fourth hour toward the horizontal until the 18h hour of incubation After this time the percentage of mitotic cells decreased abruptly

In all five cell lines in which colcemid caused a mitotic arrest a slight increase in the number of prophases was usually seen This increase however remained very small during the first 3–4 hours

In the ELT cultures both arrested metaphases and ski anaphases could be seen during the whole period of colcemid treatment but no accumulation of mitotic figures above the initial level was obtained (Fig 8) On the contrary during the initial three hours a depression



Fig 9
Micronuclei formation in colcemid treated C,H M culture

was seen. It should be emphasized that similar results were obtained with three different concentrations of colcemid, *i.e.* 0.05, 0.1 and 1.0 µg/ml.

Signs of colcemid intoxication were seen in all cultures after some time of incubation. Thus micronuclei appeared after 6–8 hours of colcemid treatment (Fig. 9). After 18–20 hours most nuclei were found *to be broken up into numerous micronuclei.* Besides this sign of colcemid intoxication, the ELT cells showed vacuolization of the cytoplasm with the formation of signet ring cells, pycnosis and karyorrhexis already after 1–2 hours of treatment.

Table 2 shows the mean values of the mitotic indices counted after 1–6 hours of colcemid treatment, *i.e.* before the appearance of micronuclei. The table also shows the means of the corresponding RT values calculated according to the formula $RT = \frac{100t}{Ct}$ where t is the time of colcemid treatment and C the mitotic index at time t. As it is seen, the values obtained after 2–4 hours of colcemid treatment were fairly constant except in the case of the LLT cells.

TABLE 3

Replication Time Determined by Three Different Methods for Six Murine Cell Lines Propagated in Vitro

Cell line	Replication time (hours) Colcemid method	Autoradiographic method	Cell count method
C_3H E	26.7 ± 3.2	25.0	30.0 ± 3.5
C_3H M	28.7 ± 2.3	25.0	29.0 ± 3.5
C_3H L	30.5 ± 4.3	26.0	26.6 ± 3.1
L-929	27.8 ± 2.4	23.5	30.5 ± 5.3
FLD	25.9 ± 1.7	22.0	25.5 ± 2.0
ELT	not determinable	20.0	23.5 ± 3.2

Median values with the 95 per cent confidence interval based on mitotic counts after 2–4 hours of colcemid treatment and cell counts for 5 consecutive days.

The RT values determined by the colcemid method, by means of autoradiography and by daily cell counts on five consecutive days are *compared in Table 3. It is seen that the RT values determined by the* autoradiographic method are 6–16 per cent lower than those determined by the colcemid method, while RT determined by cell counts showed slightly higher values in three cell lines and slightly lower values in two cell lines. These differences were not statistically significant. It is also seen that neither the colcemid method nor the cell count method revealed any significant differences between the replication time of the cell lines investigated.

DISCUSSION

The great advantage of the colcemid method for RT determinations is its rapid and uncomplicated procedure as compared with the complicated autoradiographical technique and the time consuming cell counts for several consecutive days. However, various sources of error must be borne in mind when the colcemid method is used.

First of all it is essential that the mitotic activity of the cell population under investigation is asynchronous and that colcemid has no influence on the ability of the cells to enter into mitosis. Second, all dividing cells in the population should be sensitive to the cytostatic effect of colcemid and this effect should be sufficiently long to allow a significant accumulation of mitotic figures. If the cytostatic effect is equal to or longer than the replication time, the percentage of arrested metaphases plotted against the duration of colcemid treatment will follow a linear curve reaching 100 per cent provided that all the cells are capable of division. However, *Stubblefield* (1964) has demonstrated by cinematography that the metaphase arrest in colcemid treated cultures only lasts about 12 hours. Thus, the accumulation of 100 per cent of arrested metaphases is only to be expected in cultures with a very short replication time. Third, the difference between the cytostatic and the cytotoxic effect of colcemid should be sufficiently pronounced to allow a significant number of arrested metaphases to accumulate before signs of cellular degeneration are seen.

Only four of the present cell lines met with these requirements. In the C_3H F cultures a linear accumulation of mitotic figures was observed, starting from the initial value at the time of colcemid addition to the cultures. However, after 4 hours of incubation an increase of the rate at which mitotic figures accumulated was seen. This increase was partly due to an increase of prophases, but mostly to an increased accumulation of metaphases.

In Table 2 the replication time was calculated on the basis of the mitotic indices counted after 1–6 hours of incubation. In the case of the C_3H F cells, these indices represent the sum of the number of prophases and metaphases at the beginning of the experiment plus the number of accumulated mitotic figures. Thus, the initial number of prophases and metaphases at time zero ought to be subtracted from the indices on the basis of which RT is calculated. If this is done the following mean values for RT are obtained: 30.3, 46.5, 41.1, 38.8, 25.2 and 25.9 hours after 1, 2, 3, 4, 6, and 8 hours, respectively.

These results could be explained by a partial synchronization of cell division in the C_3H F cultures. In previous experiments (*Sawicki et al.* 1967) mitotic counts at the beginning of the experiments and after 18 and 38 hours of incubation did not show any sign of synchronization, but this does not exclude a short lasting synchronization of C_3H F cells induced by colcemid before metaphase arrest.

Puck & Steffen (1963) found that colcemid had no effect on the G_1, S or G_2 stages of S3 HeLa cells. On the other hand *Figsti et al.* (1957) reviewed observations indicating a stimulatory effect on the cellular processes which initiate cell proliferation. In the present experiments no significant deviations of the prophase curves occurred during the first 3–4 hours of incubation, as one would have expected if colcemid had an inhibiting or promoting effect on cells preparing for mitosis.

This, however, does not exclude that colcemid might have a synchronizing effect on cells in prophase or pro-metaphase. *Puck & Steffen* (1963) have presented evidence that colcemid is specifically prevented from acting on S3 HeLa cells which have already entered into mitosis. The evidence was mainly based on the presence of a lag in the action of colcemid followed by a linear accumulation of mitotic figures which extrapolated back to the zero point. Similar observations were made in the present experiments with the C_3H M, C_3H L, L-929 and ELD cells, but the C_3H E cells did not show any lag in the action of colcemid. Therefore, the possibility exists that the deviation of the C_3H E curve after 3–4 hours of incubation is due to the partial synchronization of cells in prophase or prometaphase.

The C_3H M, C_3H L, L-929 and ELD cultures all showed the lag period mentioned above followed by a linear accumulation of mitotic figures until the fourth hour of incubation. This portion extrapolated back close to the zero point, indicating that colcemid in these four cell lines did not act on cells which at the beginning of the experiment had already entered into mitosis. Thus, no correction is required for the number of mitosis present in these cultures at the time of colcemid addition.

As in the case of the C_3H E cells, the prophase curves remained almost horizontal during the first 3–4 hours of incubation. After that time a moderate increase was seen, suggesting that colcemid even in these 4 cell lines might have a slight effect on cells which have already entered into mitosis. However, the increase of the number of prophase did not accelerate the total rate of accumulation of mitotic figures. On the contrary, after the fourth hour when 13.7–17.0 per cent of the cells were in mitosis the curves started to bend toward the horizontal, and after 18 hours a decline of the mitotic index was seen. Similar observations were made by *Puck et al.* (1964) in their studies of the Chinese hamster ovary grown *in vitro*. Their experiments showed that this course of the curve was due to degeneration of the mitotic cells.

In the ELT cultures addition of colcemid led to a decrease of the mitotic index during the first 3 hours of incubation. Cytological signs of cell degeneration were seen at the same time even at a concentration of colcemid as low as 0.05 µg per ml. The decrease did not continue, which indicates that the ELT cells were not all equally sensitive to the cytotoxic effect of colcemid. However, even after 12 hours of incubation the mitotic index had only reached the initial value.

The other five cell lines were more resistant to the toxic effects of

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In Table 2 the replication time was calculated on the basis of the mitotic indices counted after 1–6 hours of incubation. In the case of the C_3H F cells, these indices represent the sum of the number of prophases and metaphases at the beginning of the experiment plus the number of accumulated mitotic figures. Thus, the initial number of prophases and metaphases at time zero ought to be subtracted from the indices on the basis of which RT is calculated. If this is done the following mean values for RT are obtained: 30.3, 46.5, 41.1, 38.8, 25.2 and 25.0 hours after 1, 2, 3, 4, 6 and 8 hours, respectively.

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sitivity to colcemid intoxication On the contrary malignant cells derived from the same tumour such as the near diploid ELD and the near tetraploid ELT cells showed great difference in their sensitivity to colcemid

Thus from the present investigation it may be concluded that the colcemid method does not give more information than the cell count method It is a rapid method for the determination of the doubling time but the cell count method is safer if the effects of colcemid on the cell line under investigation are unknown The colcemid method did not reveal any differences between the growth kinetics of non malignant and malignant cells and no general correlation between malignancy and the sensitivity of the cells to the cytotoxic effects of colcemid could be established

SUMMARY

The replication time of six non malignant or malignant murine cell lines grown *in vitro* was determined by mitotic counts in colcemid arrested cultures by pulse labelling experiments with triated thymidine and autoradiographic technique and by daily cell counts for 5 consecutive days In four of the six cell lines the colcemid method was found to be a quick and reliable method if the mitotic counts were made after 2–4 hours of colcemid treatment Its only advantage over the cell count method was its shorter duration but various sources of error impose more restrictions to its use than to that of the cell count method

One cell line was found to be too sensitive to the cytotoxic effect of colcemid to allow this method for the determination of the replication time while another cell line showed signs of synchronization which made the colcemid method unsuitable The experiments did not reveal any significant differences between the replication times of non malignant and malignant cells

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AMYLOIDOSIS INDUCED IN MICE BY TRANSPLANTATION OF CASEIN SENSITIZED AND NOT SENSITIZED SPLEEN CELLS

By

OLE WERDELIN and POUL RANLØV

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In a recent paper we have described a new method of inducing amyloidosis experimentally (*Werdelin & Ranløv* 1966). In principle this method consists in transfer of spleen cells from mice with casein induced amyloidosis to syngeneic recipients. The recipient mice develop spleen amyloidosis in the course of three to four days. Autoradiographic studies of the fate of intravenously injected spleen cells from casein treated mice in syngeneic recipients have shown that such cells end up primarily in the spleen of the recipients (*Ranløv & Werdelin* 1967) and thus lend support to the possibility that the injected cells are concerned locally in the production of amyloid in the new host.

In the experiments cited above the recipient mice developed amyloidosis only when the donor mice contributing to the cell suspension had spleen amyloidosis. Thus it could not be completely ruled out that the lesions found in the recipient mice were due to phagocytosis of small particles of preformed donor amyloid.

It would be of interest therefore to find out if the transfer of spleen cells from casein treated mice without amyloidosis may promote amyloidosis in recipient mice. In the present experiment spleen cells from mice receiving casein treatment are transferred at an early preamyloidotic stage of the amyloidosis induction. After the cell transfer the recipient mice are treated with casein for a short period and finally with nitrogen mustard.

MATERIAL AND METHODS

Male and female mice 8–16 weeks of age, all belonging to the same inbred strain of C3H mice were used. All animals were selected from a large number of cages and distributed at random in the different groups. Below the methodological principles are outlined. For a more detailed account of the methods used see *Werdelin & Ranløv* (1966).

Donor treatment. 20 mice were treated with hypodermic injections of 5 per cent sodium caseinate prepared according to *Christensen* (1963) 6 days a week. Two hours

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after the tenth injection the donors were killed by cervical dislocation. All donor spleens were removed and pooled in Ringer solution. A small piece was taken from each to serve as a control for the development of amyloidosis.

Spleen cell suspension. The suspension was made by means of a loose fitting glass homogenizer. The cells were washed three times in fresh Ringer solution and the viability of the nucleated cells was determined by the trypan blue test and found to be 85 per cent. The number of nucleated cells per ml of suspension was counted in a Turl counting chamber and the suspension was adjusted to 250 × 10^6 nucleated cells per ml.

Recipient treatment. 0.4 ml of the suspension containing 100 × 10^6 nucleated cells was injected in the tail vein of each recipient mouse. All recipients were then treated with daily subcutaneous casein injections for 7 days beginning two hours after the cell transfer. 24 hours after the last casein injection treatment with nitrogen mustard was started. 0.05 mg of nitrogen mustard (Erasol IDO8) was given every second day as subcutaneous injections. 5 days after the start of the nitrogen mustard treatment, the recipients were killed by cervical dislocation and autopsied.

Control mice. One group of mice received intravenously 0.4 ml = 100 × 10^6 spleen cells of a suspension from normal mice. A second group received 0.4 ml of Ringer solution intravenously instead of cells. After the inoculation, the mice in both groups were treated with casein for 7 days followed by nitrogen mustard for 5 days.

Histological methods. Spleen, liver, kidney, adrenal, small intestine, thymus, mesenteric lymph node and lung were taken for microscopy. After fixation in neutral formalin and embedding in paraffin, sections were stained with haematoxylin-eosin, Congo red, methyl violet, pyronin-methyl green, periodic acid-Schiff stain and Thioflavine T.

Evaluation of histological lesions. A quantitative estimation of the spleen and liver amyloidosis was undertaken. The quantitation of the spleen amyloidosis in degrees 1 to 6 was carried out according to the principles of Christensen & Hjort (1959) and the liver amyloidosis was graded from 1 to 4, degree 1 meaning a few small precipitates of amyloid at the periphery of the lobule, 2 moderate perilobular amyloidosis with masses extending from the periphery some way into the lobule, 3 extensive lobular amyloidosis with escape of centrilobular area, and 4 meaning extensive amyloidosis in central as well as perilobular regions.

The histological material from all groups was screened and graded blindly twice. In cases of disagreement all sections from the animal concerned were revised and the extent and degree of amyloidosis determined.

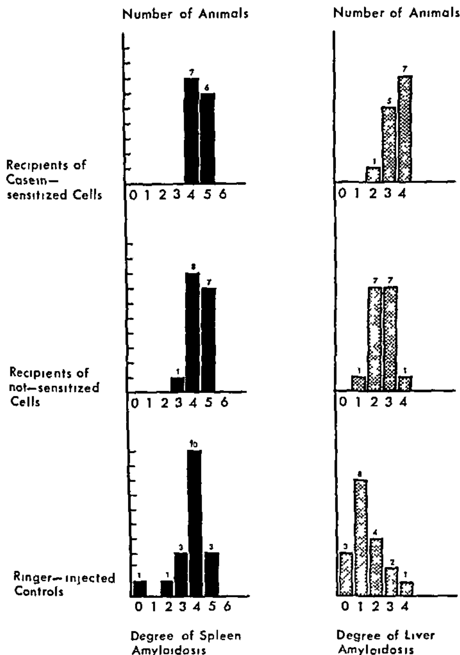
RESULTS

Microscopy of the donor spleens showed absence of amyloidosis in all cases. Members of all three recipient groups developed amyloidosis. The incidence, topography and severity of the lesions are tabulated in Table 1 and Fig. 1.

TABLE 1

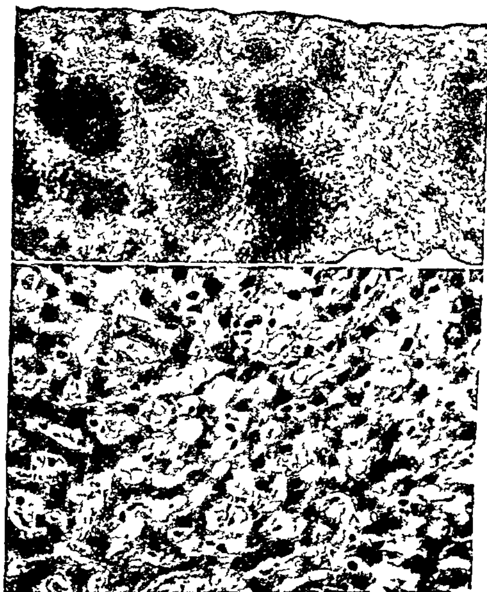
Incidence and Topography of Amyloidosis in Recipients of Casein Sensitized Cells and of not Sensitized Cells and in Ringer Injected Control Mice. All Recipients Received an Additional Treatment with Casein and subsequently with Nitrogen Mustard

Number and type of transferred cells	Incidence of Amyloidosis					
	Spleen	Liver	Lung	Kidney	Adrenal	Intest.
100 × 10^6 Casein sensitized	13/13	13/13	13/13	13/13	10/10	3/11
100 × 10^6 Not sensitized	16/16	16/16	16/16	13/16	5/11	1/16
None (Ringer injected)	17/18	15/18	0/18	1/17	0/15	0/17



F 1

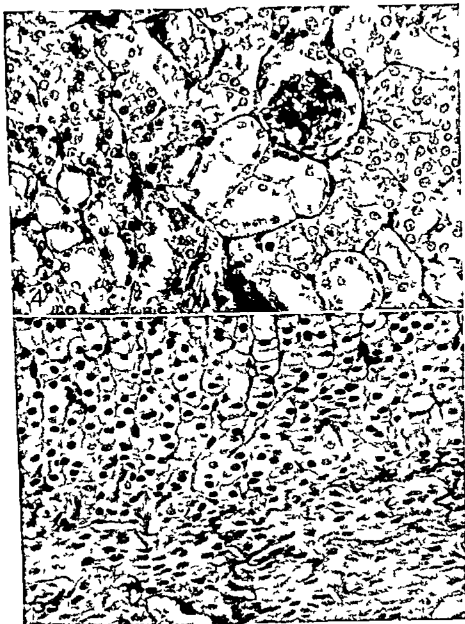
Degree of spleen (left column) and liver amyloidosis (right columns) in recipients of spleen cells from casein treated donors (upper columns), in recipients of cells from not sensitized donors (middle columns) and in Ringer injected controls (lower columns). All recipients were subsequently treated with an additional course of 7 days of casein injections followed by 5 days of nitrogen mustard



Figs 2, 3

Fig 2 Spleen from recipient of 100 × 10 casein sensitized spleen cells Amyloidosis degree 5 Periodic acid Schiff stain × 3[illegible]

Fig 3 Liver from recipient of 100 × 10[4] spleen cells from casein sensitized donor mice Kupffer cells distended with amyloid Haematoxylin eosin × 350



Figs 4 5

Fig 4 Kidney from recipient of 100 × 10 spleen cells from casein sensitized donors Deposit of amyloid in the glomerular tuft Periodic acid Schiff stain × 350

Fig 5 Adrenal gland from recipient of spleen cells from casein sensitized donor mice Deposits of amyloid in the boundary zone between cortex and medulla with extensions into the cortical zona reticularis Periodic acid Schiff × 350

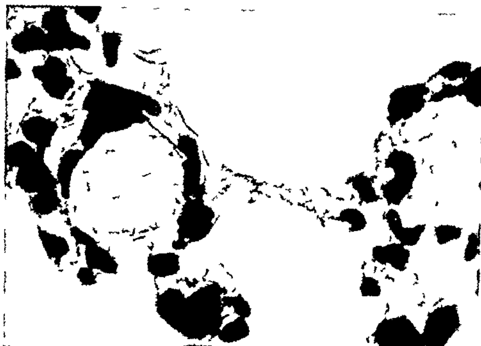


Fig 6
Lung from recipient of 100 × 10⁶ spleen cells from casein sensitized donors Mass of amyloid in alveolar wall Haematoxylin eosin × 1400

The spleen amyloidosis presents the usual pattern of rings surrounding the splenic follicles (Fig. 2) The degree of amyloidosis in recipients of cells is higher than that in Ringer injected controls

The liver amyloidosis seems to start at the periphery of the liver lobule and to spread towards the central vein The earliest trace of amyloid can be detected around the vessel walls in the periportal space at the periphery of the lobule In more advanced lesions the precipitates can be traced in the sinusoidal walls where a deposit often shows as a *round globule expanding the Kupffer cell the nucleus of which* is found riding on the top of the cell towards the sinusoid (Fig. 3) A definite quantitative difference between the three groups is obvious *recipients of casein sensitized* cells showing very pronounced liver amyloidosis and Ringer injected controls slight liver amyloidosis only Recipients of not sensitized cells develop liver amyloidosis of a degree between these two groups

Amyloid was found in the kidneys of recipients of cells but not in *Ringer injected controls In recipients of casein sensitized cells* small masses of amyloid were found in individual coils of the glomerular tuft and interstitial amyloidosis in the form of oblong deposits was pronounced (Fig. 4) Recipients of not sensitized cells showed slight interstitial amyloidosis but glomerular lesions were absent

In the adrenal gland amyloid was *found* around the vessels in the

cortico medullary junction (Fig. 5) From here amyloid can be traced a short distance out into the cortex all deposits lying in close relation to the vessels In the Ringer injected controls adrenal lesions were absent

Amyloid was found in the intestinal wall of a few of the recipients of cells The amyloid was located around the small vessels lying at the *base of the crypts of Lieberkuhn and deposits were also found around* the central vessel of the intestinal villi

In the lungs of recipients of cells were found small round or oval bodies of PAS positive amyloid like material lying in the alveolar capillaries and lung arterioles (Fig. 6) Amyloid was not found in the thymus in any animal In the mesenteric lymph node from some recipients of cells was found very small deposits of amyloid around vessels of the pulp only detectable in Congo stained sections through crossed polars

Periodic acid Schiff stained sections revealed the occurrence of cells with PAS positive cytoplasm in and around the splenic deposits of amyloid Many of the PAS positive cells have oval or kidney shaped vesicular nuclei and granular cytoplasm and resemble reticular cells (*Teilum* 1956) The histochemical reactions of the amyloid in different localizations are the same and correspond to the histochemistry of mouse amyloidosis induced by other means The amyloid is PAS positive *and Congo positive It gives a bright yellow fluorescence in Thio* flavine T stained sections in ultraviolet light and displays green birefringence with Congo red in polarized light The amyloid stains orthochromatic with methyl violet

DISCUSSION

In the present experiment spleen cells have been transferred at an early preamyloidotic stage of the casein treatment After the transfer the recipient mice receive casein treatment for a period before the nitrogen mustard treatment This experimental system of course excludes the possibility of transfer of particles of amyloid to the recipients who nevertheless develop a far more pronounced and widespread amyloidosis than Ringer injected controls Thus it can be safely concluded that the amyloidosis promoting effect of the cell suspension is not caused by preformed donor amyloid Inoculation of spleen cells from normal mice also led *to severe amyloidosis However a comparison* with the results of inoculation of spleen cells from casein sensitized mice reveals a definite quantitative difference In the latter group the liver amyloidosis is more severe and the incidence and degree of extrasplenic and extrahepatic amyloidosis is higher

The development of amyloidosis in Ringer injected control mice is the result of casein treatment for 7 days followed by treatment with nitrogen mustard for 5 days When the post transfer casein treatment

is reduced to 4 days. Ringer injected controls do not develop amyloidosis while the majority of spleen cell inoculated recipients develop spleen amyloidosis (*Werdelin & Ranløv* 1966 unpublished). Experiments which may have some bearing on the amyloidosis enhancing effect of transplantation of tissues and cells was carried out by Letterer already in 1926 (*Letterer* 1926). In order to see if once formed amyloid can be resorbed Letterer transplanted pieces of amyloidotic rabbit spleens to the peritoneal cavity of allogeneic recipients. Somewhat surprisingly a number of these developed spleen and liver amyloidosis in the course of weeks. Grafting of liver, kidney and brain from normal rabbits had the same effect on allogeneic recipients independently of the condition of the graft whether viable or necrotic. In our experiments (*Werdelin & Ranløv* 1966 unpublished) we have tried implantation of whole amyloidotic mouse spleens in the peritoneum of syngeneic recipients without being able to transfer the amyloidosis. The time from the transplantation to death was only 6 days during which the mice were treated with nitrogen mustard.

More recently *Barnes et al* (1962) and *Bradbury & Micklem* (1965) have demonstrated the development of amyloidosis in mouse radiation chimeras. After lethal irradiation the mice had been restored with syngeneic foetal liver cells or by allogeneic bone marrow cells. The chimeras developed spleen and liver amyloidosis in the course of 20 to 120 days. It was emphasized that functional exhaustion of the immune mechanisms of the chimeras might play an important role in the development of the amyloidosis. This view is consistent with that of *Teilum* (1964) according to which amyloid is produced by cells of the reticulo-endothelial system exhausted during a prolonged antigenic stimulation.

The amyloidosis resulting from the spleen cell transfer and the following treatment in the present experiment is remarkable for its severity. The role played by the transferred cells remains to be explained. In an earlier paper (*Werdelin & Ranløv* 1966) it was suggested that the transferred cells having colonized the recipient are involved locally in the synthesis of amyloid under the influence of nitrogen mustard which is a potent amyloidosis-enhancing drug (*Teilum* 1964). This suggestion was supported by autoradiographic investigations (*Ranløv & Werdelin* 1967) in which H^3 labelled spleen cells from casein treated mice were traced to the recipients spleens and lungs the actual sites of amyloidosis.

Do the transferred cells however migrate to all the sites where amyloid is found in the present experiment? Investigations of the fate of intravenously injected lymphoid cells show little difference of opinion regarding the early events after the cell transfer (*Osogoe* 1950, *Weisberger et al* 1951, *Keohane & Metcalf* 1958, *Diderholm* 1961). Many cells are trapped in the lung capillaries shortly after the injection. *Most of them are quickly released* into the blood stream and in

creasing numbers are found in the spleen during the following 24–48 hours Actually the spleen is the place where the highest concentration of transferred cells is found at any time (*Hattler et al* 1964 *Murray & Murray* 1964) Concomitantly with the colonization of the spleen labelled cells can be found in the liver lymph nodes Peyers plaques of the intestine and the kidneys and migration to the intestinal mucosa has been observed by several investigators (*Farr* 1951 *Ambrus & Ambrus* 1959 *Shorter & Bollman* 1960) Thus the literature affords evidence that transferred lymphoid cells may migrate to the locations where amyloid is found in the present transplantation experiment with the exception of the adrenal which does not seem to have been subjected to lymphokinetic studies with labelled cells

SUMMARY

An investigation has been undertaken in order to find out if the amyloidosis promoting effect of syngeneic transplantation of spleen cells from mice with casein amyloidosis is dependent on the presence of amyloid in the transferred cell suspension

In the present experiment is demonstrated an amyloidosis promoting effect of the transfer of spleen cells from casein sensitized donors without amyloidosis and although weaker of transfer of spleen cells from normal mice

The parallelity between the distribution of intravenously injected lymphoid cells and the topographic distribution of the amyloid is emphasized The possible participation of transferred cells in a local synthesis of amyloid is discussed

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Acta path et microbiol scandinav 7 23–30 1968

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AMYLOIDOSIS INDUCED BY CELL TRANSFER

Effects of Heat Damaged and of X Irradiated Spleen Cells from Casein Sensitized Mice

By

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Received 5 xi 67

An amyloidosis promoting effect of syngeneic transplantation of spleen cells has been shown on mice by different experimental procedures by transplantation of spleen cells from mice with casein amyloidosis to nitrogen mustard treated recipients (*Werdelin & Ranlov* 1966) by transplantation of spleen cells from casein sensitized mice without amyloidosis to recipients treated with casein + nitrogen mustard and by transplantation of spleen cells from normal mice to recipients treated with casein + nitrogen mustard (*Werdelin & Ranlov* 1967)

On the basis of autoradiographic investigations of the fate of labelled intravenously transferred casein sensitized cells (*Ranlov & Werdelin* 1967) and the literature on the fate of intravenously injected lymphoid cells (for a review see *Diderholm* (1961) *Keohane & Metcalf* (1958) *Shorter & Bollman* (1960) *Cowans* (1962) *Murray & Murray* (1964) it was emphasized that the topographic distribution of the amyloid in transfer experiments parallels that of injected cells It was suggested that the transferred cells might in some way be concerned in the synthesis of amyloid locally The nature of the role played by the transferred cells however needs further elucidation

It was decided that the amyloidosis promoting effect of spleen cells should be investigated to see whether it is A) thermo resistant and B) dependent on the proliferative capability of the cells In experiment A the amyloidosis promoting effect of heat damaged spleen cells is compared with that of not damaged and X irradiated spleen cells from the same group of amyloidotic donors Experiment B was designed in order to determine if proliferative capability of the transferred cells is significant for the events after the cell transfer The amyloidosis promoting effect of X irradiated and not irradiated spleen cells from the same group of casein sensitized mice without amyloidosis is compared

MATERIAL AND METHODS

Male and female CBH mice 6–24 weeks old of the same inbred strain were used throughout the experiments. The general methods used have been the same as in previously published experiments (Werdelin & Ranløv 1966) unless otherwise described. At the start of each experiment donor and recipient mice were selected at random from a large number of cages and put in new cages, 4–8 together.

Donor mice received one daily injection six times a week of 0.5 ml of 5 per cent sodium caseinate prepared according to *Christensen (1963)*. After the casein treatment the donor mice were killed by cervical dislocation and the spleens pooled in Ringer solution. All the spleens were split along the long axis and from each a small piece was taken for microscopy serving as a control for the development of amyloidosis.

Spleen cell suspension was made by means of a loose fitting glass homogenizer. The crude suspension was filtered through a fine meshed metal sieve (1600 holes per cm²). The filtrate containing the cells was centrifuged for 3 minutes at 1500 rounds per minute and then resuspended in fresh Ringer's solution. The washing was repeated twice. Finally the number of nucleated cells per ml suspension was counted in a Türk counting chamber and the suspension was adjusted to the desired number of cells per ml. The viability of the nucleated cells was controlled by the trypan blue test.

Recipient mice were in the natural state at the time of the cell transfer. The stipulated number of cells was introduced by injection in the tail vein. After the cell transfer the treatment of the recipient mice with casein or nitrogen mustard was initiated within 1–3 hours.

Autopsy and histological methods. The recipient mice were killed by cervical dislocation. From each animal was taken spleen, liver, lung, thymus, kidney, adrenal, small intestine and mesenteric lymph node for microscopy. After fixation in neutral formalin and embedding in paraffin sections were stained with haematoxylin-eosin, pyronin methyl green, Congo red and periodic acid Schiff stain.

Quantitation of the spleen amyloidosis was carried out blindly on the PAS stained sections using the grading of *Christensen & Hjort (1961)*. Spleen amyloidosis is graded from 1–6: degree 1 expresses a few incomplete amyloid rings around the splenic follicles, degree 2 thin complete rings of amyloid, degrees 3–5 rings of increasing breadth, and degree 6 the maximal degree of amyloidosis with obliteration of splenic structure.

The details of the two experiments are given below.

A. *Transfer of Heat Damaged Spleen Cells from Mice with Casein-amyloidosis to Recipients Treated with Nitrogen Mustard*

40 donor mice were treated with 14 casein injections. All donors were killed on the day of the last casein injection. The spleen cell suspension adjusted to 2.[illegible] × 10^{8} cells per ml was divided into 3 portions: a) was heated to 5[illegible] °C for 30 minutes, b) was exposed to a dose of 1000 r using a Siemens "Stabilivolt" X-ray machine (100 kV, 12 mA, HVL of 0.09 mm Cu, dose rate 5[illegible] r/min) and c) was not treated. 15 mice each received intravenously 0.36 ml = 100 × 10^{6} cells from suspension a, 15 mice each received 0.36 ml = 100 × 10^{6} cells from suspension b and 19 mice each received 0.36 ml = 100 × 10^{6} cells from suspension c. 1 to 3 hours after the cell transfer each recipient received an injection of 0.05 mg of nitrogen mustard (Bras[illegible] IDO?) subcutaneously. This was repeated 48 and 9[illegible] hours after the cell transfer and all recipient mice were killed 120 hours after the cell transfer. The spleen was lost at autopsy from one recipient of suspension a and from two recipients of suspension c. These animals were discarded from the groups.

B. *Transfer of X-irradiated Spleen Cells from Casein Sensitized Donors without Amyloidosis to Recipients Treated with Casein + Nitrogen Mustard*

30 donor mice were treated with 7 casein injections and killed on the day after the last casein injection. The spleen cell suspension was adjusted to 1[illegible] × 10^{8} cells per ml. One half of the suspension was irradiated with [illegible]000 r generated by a Siemens "Stabilivolt" X-ray machine (100 kV, 1[illegible] mA, HVL of 0.09 mm Cu, dose rate 59 r/min), the other half was not irradiated or damaged by other means. 14 mice each

received 0 3 ml = 50 × 10⁶ irradiated spleen cells and 17 mice each received 0 3 ml = 50 × 10⁶ not irradiated cells A third group of 18 mice each received 0 3 ml Ringer solution i v ¼–1½ hours after the cell transfer the members of all three groups received the first casein injection repeated 24 72 and 96 hours later 24 hours after the last casein injection treatment with nitrogen mustard was initiated 0 05 mg was given every second day for 5 days whereupon all recipient mice were killed and autopsied

RESULTS

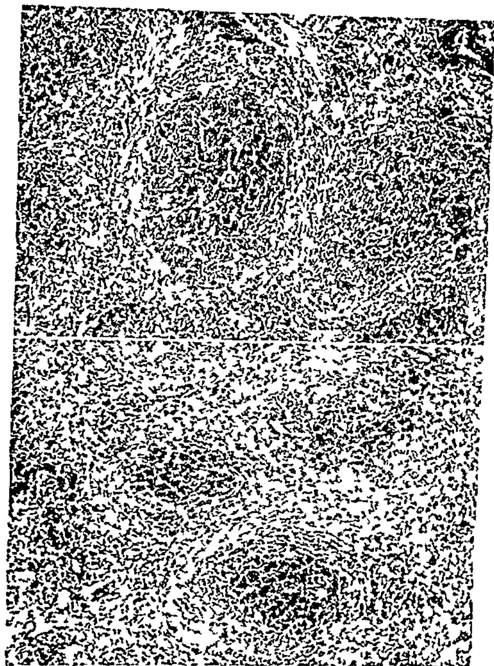
Transfer of Heat Damaged Spleen Cells from Mice with Casein Amyloidosis to Recipients Treated with Nitrogen Mustard

Among the 40 donor mice 15 had developed spleen amyloidosis The viability of the heated cell suspension was 70 per cent and that of the not damaged and irradiated suspensions was 85–90 per cent The result of the transfer experiment is shown in Table 1 Recipients of heat damaged cells develop spleen amyloidosis with approximately the same frequency and intensity as the groups inoculated with not damaged and irradiated cells from the same group of donors The spleen amyloidosis is of degrees 1 to 3 and presents the usual pattern of rings of homogeneous substance surrounding the splenic follicles (Figs 1 and 2) Histochemically it is PAS positive Congo positive and it shows green birefringence in Congo stained sections in polarized light In the lungs of all recipients are found many small lumps of PAS positive amyloid like material in the alveolar capillaries and small arterioles Sections of liver and kidney were available from all recipients No amyloid was found except small precipitates in the liver of one recipient of X irradiated cells Intestine adrenal gland mesenteric lymph node and thymus were available from the majority of the recipients and no amyloid was found

TABLE 1

Incidence and Degree of Spleen Amyloidosis in Nitrogen Mustard Treated Recipients of Heat Damaged X Irradiated and Not Damaged Spleen Cells from Mice with Casein Amyloidosis

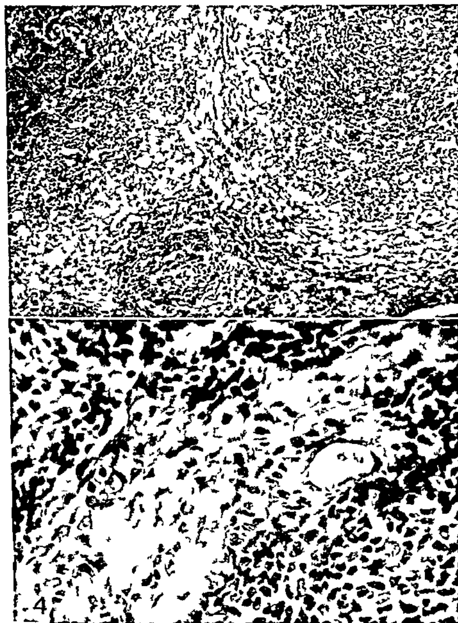
Dose and type of spleen cells	Number of recipients with spleen amyloidosis of			Number of recipients with spleen amyloidosis/total number of recipients in group
	degree 1	degree 2	degree 3	
100 × 10⁶ heat damaged	3	5	1	9/14
100 × 10⁶ not-damaged	2	10	1	13/17
100 × 10⁶ X irradiated	0	11	0	11/15



Figs 1-2

Fig 1 Spleen from recipient of 100 × 10^6 spleen cells from casein sensitized donors with amyloidosis. *Recipient treated with nitrogen mustard for 5 days.* Spleen amyloidosis degree 2. Periodic acid Schiff stain × 140

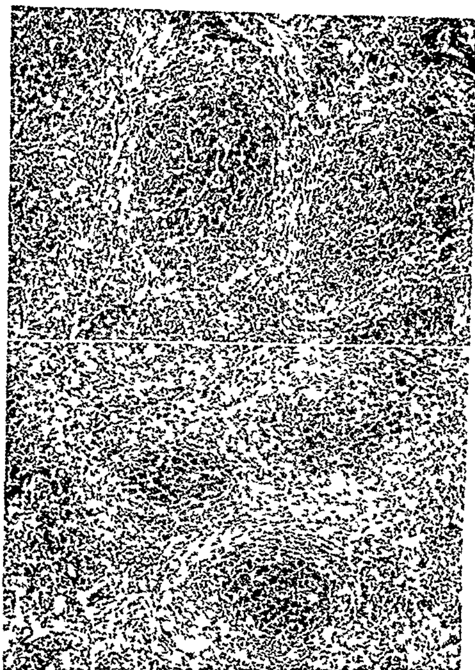
Fig 2 Spleen from recipient of 100 × 10^6 heat-damaged spleen cells from casein sensitized donors with amyloidosis. Recipient treated with nitrogen mustard for 5 days. Spleen amyloidosis degree 3. Periodic acid Schiff stain × 140



Figs 3–4

Fig 3 Spleen amyloidosis of degree 4 Recipient of 50×10^6 X irradiated spleen cells from casein sensitized donors Recipient treated with casein for 7 days and nitrogen mustard for 5 days subsequent to the cell transfer Periodic acid Schiff stain × 140

Fig 4 Same recipient as Fig 3 Detail of amyloid in the perifollicular zone of the splenic white pulp Reticular cells in the amyloid substance Periodic acid Schiff stain × 560



Figs 1–2

Fig 1 Spleen from recipient of 100×10^6 spleen cells from casein sensitized donors with amyloidosis Recipient treated with nitrogen mustard for 5 days Spleen amyloidosis degree 2 Periodic acid Schiff stain $\times$ 140

Fig 2 Spleen from recipient of 100×10^6 *heat damaged spleen cells from* casein sensitized donors with amyloidosis Recipient treated with *nitrogen mustard* for 5 days Spleen amyloidosis degree 3 Periodic acid Schiff stain $\times$ 140

Measured by the trypan blue test the viability of the transferred cells in the present experiments ranges between 75 and 85 per cent However cell viability as determined by the trypan blue test does not mean that the cells will be able to proliferate or that they have retained all their metabolic activities *Tennant* (1964) found that the cells in cultures irradiated with 2000 r was not able to divide although 90–95 per cent remained unstained by the dye Cell suspensions heated to 60 C for 3 minutes showed no evidence of metabolic activity, and cultivability was reduced to 1 per cent although the trypan blue viability ranged between 80 and 90 per cent

From experiment A it seems justified to conclude that the amyloidosis promoting effect of the transferred spleen cells does not depend on heat labile metabolic activities in the cells In experiment B the conditions for proliferation of the transferred cells are favourable during the post transfer treatment Nevertheless the amyloidosis enhancing effect of roentgen killed cells is the same as that of not irradiated cells i e it is not dependent on the capability of the cells to proliferate in the new host

As emphasized in earlier communications (*Werdelin & Ranløv* 1966 1967 *Ranløv & Werdelin* 1967) the transferred spleen cells are sequestered in the same areas as the developing amyloid This supports the possibility that the transferred cells may be involved locally in the synthesis of amyloid In view of the present findings the role of the transferred cells seems to be a passive one while the part played by the recipients own reticulo endothelial cells remains to be defined

SUMMARY

The amyloidosis enhancing effect of transplantation of spleen cells from casein sensitized mice to syngeneic recipients has been compared with that of roentgen killed and heat damaged cells from the same groups of donors

Irradiation with 1000 r or 2000 r or heating to 56° C for 30 minutes does not weaken the amyloidosis enhancing effect of the spleen cells

It is concluded that the amyloidosis enhancing effect of syngeneic transplantation of spleen cells from casein sensitized mice is not dependent on heat labile metabolic activities in the transplanted cells or their capability to proliferate

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THE CAUSES OF PERINATAL DEATH

By

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In recent years interest in perinatal mortality has been steadily increasing in a number of countries (1 5 8 9 10 12 14 15)

All are by no means agreed on the chief causes of the relatively high rate of perinatal mortality a rate which has not been reduced in conformity with reductions in the overall mortality rate and the advance of medical treatment

It is sometimes difficult to compare the results of individual enquiries but this depends on how far they are based on clinical or pathological anatomical investigations or on both The vital importance of autopsy in this connexion cannot be overestimated (7)

However most of those who have carried out investigations in this field agree about the important part played in perinatal mortality by anoxia congenital malformations and intracranial trauma

The present enquiry has been undertaken in order to investigate the situation obtaining in Iceland and enable comparisons to be made with conditions in other countries in fact it is the first of its kind to be made in this country

MATERIALS AND METHODS

The autopsy reports on all still born babies or babies dying soon after birth brought for autopsy during the years 1955–64 to the Department of Pathology of the University of Iceland were examined This is the only institution in the country in which investigations of this kind are carried out In addition all samples obtained from these autopsies were re examined under the microscope and the conclusions to be drawn were reviewed in the light of this examination and with reference to case histories and clinical diagnoses

A closer stud of these documents revealed th t by far the greater number of autopsies were performed on bodies from a single hospital i e the Obstetric and Gynaecologic Department of the *Lan lsp tali* (the University Hospital) in Reykjavik which is far the largest Obstetric department in the countr and the one with the best facilities for the ad anced treatment of matern ty ase nd therefore the one to which the most difficult cases are sent

To obtain a summary of the number f li ng i tl t ll b rths and early neonatal death during the period in question th b th r ls f th hospital were examined These sho ed that nearly 50 per cent f th t ll l n c es and 90 per

The author wishes to convey his gratitude to the head of the Obst and Gynecolog Dep tm t t Profe r *P H J Jakobsson* for his kindne in allowing free access to th h pital records

cent of cases of death within six days of birth were sent to the Department of Pathology for autopsy The majority of still born babies not sent for autopsy or 65 per cent were very premature (weight 1001–2000 g) and *major or minor signs* of macerations were seen in about 50 per cent of these In the birth records the causes of death in some of these cases are also mentioned including two cases of anencephaly two of hydrocephalus and three others in which considerable malformations were present *while there are six reports of Rhesus incompatibility*

In the present enquiry the autopsies are always taken as a basis for diagnosis though account is also taken of case histories and clinical diagnoses wherever available and when there is reason for so doing

TABLE 1

Total Births Stillbirths Early Neonatal Deaths and Perinatal Mortality in Iceland

	Total births	Still births cases	Still births per 1000 births	Early neonatal death cases	Early neonatal death per 1000 births	Perinatal mortality rate per 1000 births
1951–55	21451	337	15.7	214	9.9	25.6
1956–60	24039	317	13.2	209	8.7	21.9
1961–65	23954	329	13.8	245	10.2	24.0

Total births

In Iceland the following regulations apply with regard to living and still births

A child is considered to have been born living only if it breathed of its own accord no matter for how short a time after birth and if so there is an obligation to notify it as such irrespective of how small or immature it may have been

Children that do not breath after birth are regarded as *still born* even though they may show for a short while other signs of life They are only registered as still born if the length of body is not less than 35 cm and the duration of pregnancy was 28 weeks In practice this is equivalent to a weight of 1000 g (16)

The expression perinatal mortality is used here in the restricted sense i e it includes all still births and neonatal deaths occurring within seven days of birth

TABLE

Autopsy Findings in Premature and Mature Stillbirths and Early Neonatal

Causes of death	Still births: Macerated foetus 1001–2500 grm	Macerated foetus over 2500 grm	Macerated foetus Total	Non macerated foetus 1001–2500 grm	Non macerated foetus over 2500 grm	Non macerated foetus Total
Congenital malformations	–	–	–	7	3	10
Erythroblastosis foetalis	–	5	5	–	2	2
Anoxia	6	15	21	15	28	43
Intracran trauma	–	–	–	1	10	11
Visceral trauma	–	–	–	1	1	2
Pneumonia	–	2	2	1	3	4
Hyaline membrane disease		–	–	–	–	–
Other known causes	1	–	1	1	–	1
Undetermined	5	5	10	1	–	1
Total	12	27	39	27	47	74

In the text the abbreviation Obst Dep will be used for the Obstetric and Gynaecologic Department of the Landspitali HMD will be used for hyaline membrane disease I see no reason why the term respiratory distress syndrome (RDS) should be used since the diagnosis in such cases is based chiefly on pathological rather than clinical investigation (4) On the other hand RDS is a more appropriate bedside diagnosis while HMD hardly can be diagnosed with certainty except by microscopic examination of the lungs

During the years covered by this enquiry *i e* 1955 64 15519 babies were delivered in the Obst Dep in Reykjavik that is about one third of all births in the country The national birth rate was 8 per one thousand inhabitants

Still births in the Obst Dep numbered 250 and deaths within seven days of birth 213 giving a total of 463 Hence the perinatal mortality rate was 31 0 per one thousand births During the same period the rate applying to the whole country as 23 per one thousand (Table 1) 17 18)

In the Obst Dep there were 874 premature births 5 6 per cent of the total number of births Of these premature births 139 were still births and 735 living but 127 died within seven days The death rate for premature births was thus 17 3 per cent The average rate of premature births throughout the country during this period was 5 4 per cent reducing steadily over the ten year period from 6 0 per cent to 4 1 per cent (C) The rate of premature births in the Obst Dep did not fall to the same extent owing to the fact that during the latter part of the period new obstetric departments were opened in the town and its environs so that the proportion of difficult cases including premature births dealt with there became still greater The same applies in the cases of the perinatal mortality rate

RESULTS OF AUTOPSIES

In Table 2 a summary is given of the principal diseases found at autopsy From this it can be seen that a great majority of the total number of deaths or about 58 per cent occurred in cases of premature birth but this percentage would be still greater if a substantial number of macerated prematures had not escaped autopsy as mentioned above

For the same reason the ratio of macerated to non macerated may be somewhat distorted though it should serve as rough measure of antepartum and intrapartum mortality

Deaths from the Department of Pathology University of Iceland 195 –1964

Early neonatal death				Total perinatal mortality				
1000 grm and less	1001 500 grm	over 2500 grm	Total	1000 grm and less	1001–2500 grm	over 00 grm	Total	per cent
1	11	22	34	1	18	25	44	14
–	3	6	9		3	13	1?	5
2	22	7	31	?	43	50	9	31
1	7	9	17	1	8	19	8	9
–		–	–	–	1	1	2	
?	15	3	?4	?	16	8	30	10
–	46	?	5		46			17
–	?	4	10		8	4	1?	3
11	9	–	?0	11	1		31	10
?1	119	57	197	1	158	131	310	99

From this table it may also be seen that almost one third of the deaths were due to anoxia or intrauterine asphyxia (31 per cent) Next on the list comes HMD (17 per cent) then malformation (14 per cent) and lastly pneumonia (10 per cent) and intracranial trauma (9 per cent)

HMD is found only in living births and it accounts of one fourth part of all deaths in that category

Congenital Malformations

These are grouped in separate classes in Table 3 Here of course only malformations that can be regarded as *primary causes of death* are included

Heart defects were found only in mature babies Microscopic examination revealed *haemorrhage of the lungs* in 50 per cent of these Minor heart defects such as a small *septal defect*, were not regarded as primary causes of death

Kidney defects were divided into groups of *ren cystic* and *aplasia ren* Special note was taken of the fact that three of the cases of defective

TABLE 3

Major Congenital Malformations in Different Organ Systems Sex Distribution

	Males	Females	Total
Central nervous system	6	9	15
Cardiovascular system	4	2	6
Alimentary system	6	0	6
Urinary system	5	2	7
More than one lethal malformation	4	4	8
Hernia diaphragmatica	2	0	2
Total	27	17	44

TABLE

Analysis of 95 Cases of Anoxia According to Pathologic

	Still births					
	Macerated foetus			Non macerated foetus		
	1001–2500 grm	over 2500 grm	Total	1001–2500 grm	over 2500 grm	Total
Pulm haemorrhage	2	6	8	11	10	21
Pulm haemorrhage et aspiratio	3	5	8	1	14	15
Aspiratio of amniotic detritus	1	4	5	–	2	2
Intraventricular haemorrhage	–	–				
Other intracran haemorrhage		–	–	3	–	5
Total	6	15	21	15	28	43

kidney also had *HMD* two at a very advanced stage—though the babies concerned lived for 6 hours and 9 hours respectively and were the heaviest babies among the total number with *HMD* weighing 3000 g and 3800 g. In the third case of defective kidney with *HMD* the baby lived only for 40 minutes.

Anoxia

All cases showing symptoms of *anoxia* but absence of other evident defects to which death could be attributed came into this category. Cf. Tables 2 and 4.

In all these cases haemorrhage was found to be of major or minor degree in the various organs, at least under the microscope, especially in the thymus gland, under the surface of the visceral pleura and epicardium, and above all along the coronary artery. There was a very high blood content in all organs, especially however in the spleen, liver and suprarenal gland. In addition a certain degree of haemorrhage was manifest in the lungs, visible sometimes to the naked eye, though generally only detectable by microscopy; such haemorrhage might occur either in the interalveol septa or in the actual alveoli. Associated with this was the more or less evident detritus of amniotic fluid in the alveolar spaces, together with meconium and mucus, in the greater as well as in the lesser ducts. Besides, four cases with considerable haemorrhage of the lungs, in which the lungs were completely suffused with blood, are also included in this category.

All cases of cerebral haemorrhage without visible rupture are also included in this group. Among 25 such cases haemorrhage in the cerebral ventricles was noted in 9—a diagnosis made only, however, if blood clots in one or more cerebral ventricles were found at autopsy. These cerebral haemorrhages occurred only in very small premature

...atomical Findings 1955–1964 (Cf. Tables 2 and 5)

Early neonatal death Within 7 days				Total perinatal mortality			
...00 grm and less	1001–2500 grm	over 2500 grm	*Total*	1000 grm and less	1001–2500 grm	over 2500 grm	Total
	5	5	10		18	1	39
	1	–	1		5	19	24
–	–	–	–		1	[illegible]	7
1	8	–	9	1	8		9
1	8	[illegible]	11	1	11	4	16
[illegible]		7	31		[illegible]	50	95

TABLE 5

Analysis of the 95 Cases of Anoxia According to Complications

	Still births					
	Macerated foetus			Non macerated foetus		
	1001–2500 grm	over 2500 grm	Total	1001–2500 grm	over 2500 grm	Total
Abruptio placentae	4	8	12	10	8	18
Placenta praevia	–	–	–	1	2	3
Compression of umbilical cord	–	1	1	2	8	10
Other known causes	–	4	4	–	2	2
Undetermined	2	2	4	2	8	10
Total	6	15	21	15	28	43

babies five of whom also had pneumonia while three had severe haemorrhage of the lungs In other cases of cerebral haemorrhage pneumonia was not found though severe lung haemorrhage occurred in three

In Table 5 cases of anoxia due to various complications during labour are recorded Abruptio placentae or placenta praevia as well as compression of umbilical cord were responsible in the great majority of cases accounting for more than 50 per cent of these

Traumatic Cerebral Haemorrhage

Relatively the great majority of these lesions were seen in the category Non macerated still born infants or in infants who presumably died during birth In four such cases forceps had been used

In a further six cases anoxia was involved

In the category Early neonatal deaths" intracranial trauma with haemorrhage was seen in about 9 per cent of all cases in seven cases these birth injuries were accompanied be severe haemorrhage or a presence of amniotic fluid in the bronchial ducts and alveoli In one case pneumonia was also present and in one pulmonary haemorrhage

Other birth injuries observed were ruptura lienis and ruptura gland suprarenalis *but in these cases version had been carried out on account* of faulty position of the foetus and later extraction had been performed

Pneumonia

Only cases of pneumonia are included in which the disease is regarded as the primary cause of death As mentioned before many cases of pneumonia were seen among those who apparently died of intraventricular haemorrhage but these are not included

5

at Labour 1955–1964 (Cf Tables 2 and 4)

Early neonatal death Within 7 days				Total perinatal mortality			
1000 grm and less	1001–2500 grm	over 2500 grm	Total	1000 grm and less	1001–2500 grm	over 2500 grm	Total
1	2	2	5	1	16	18	35
–	3	–	3	–	4	2	6
–	–	–	–	–	2	9	11
–	3	5	8	–	3	11	14
1	14	–	15	1	18	10	29
2	22	7	31	2	43	50	95

Hyaline Membrane Disease (HMD)

Among newborn babies HMD is the principal cause of death i e it accounts for one fourth part of all deaths occurring within the first seven days after birth Nearly three quarters of the deaths occur among babies in the weight group 1001–2000 g (Table 6)

Among babies weighing over 2500 g there were in fact 4 with immature glomeruli of the kidneys while 2 had pneumonia as well One of these had also a rupture of the falx cerebri with severe intracranial haemorrhage but owing to the size of the hyaline membrane it was included in this category and not classified as an intracranial trauma

The incidense of HMD was higher in males than females viz 29 to 23

Most babies with HMD lived for a very short time as can be seen from Table 6 The great majority died within the first twelve hours especially the smallest babies but hazards of death are really highest after the first six hours Before the first twenty four hours have elapsed 67 per cent of these babies will in fact already have died

Other Known Causes

Some rare cases are grouped here the principal being Kern icterus hydrops foetalis without erythroblastosis sclerema neonatorum necrosis hepatis tumor pleurae colitis ulcerosa + peritonitis + septicaemia thrombosis of cerebral artery + cerebral emollition

Undetermined Causes

Most of these lesions are seen in premature infants more than 50 per cent belong in the category babies weighing less than 1000 g in most cases atelectasis of major or minor degree was manifest Also three second twins and one first twin are included in this group

TABLE 6

Hyaline Membrane Disease Age Groups and Weight Groups (in Gramms)

	1001–1500	1501–2000	2001–2500	over 2500	Total
0–6 hours	5	5		1	11
6–12 hours	10	5	3	1	19
12–24 hours	2	1	1	1	5
1–2 days	2	6	2	3	13
2–5 days		2	1	1	4
Total	19	19	7	7	52

COMMENTS

In the introduction to this study the object of the enquiry was described as a search for the chief causes of perinatal death in Iceland the object being to discover whether these were the same in other countries Touching the first point the main conclusions to be drawn from the enquiry have already been described they are based first and foremost on autopsy findings supported in many cases by case histories When autopsies are carried out thoroughly and microscopic examination of organs made by trained pathologists with an interest in this particular field the diagnosis should not present many doubtful elements though in 10 per cent of the cases any conclusions could not be drawn these 10 per cent were grouped under undetermined causes

Anoxia

Anoxia in fact is the largest group in the list of lethal diseases accounting for 95 cases In the strictest sense anoxia is not a pathological concept rather it is of a physiological nature But we have no better common term for the pathological condition that underlies or accompanies the following findings

1 Minor haemorrhages in the coverings of various internal organs
2 General excess of blood in organs often with haemorrhages especially in lungs and brain
3 Lungs more or less full of deposit and meconium from amniotic fluid cf. Table 4 Reference is also made to the clinical reports on the births cf. Table 5

Admittedly the possibility of doubtful elements is always involved in these cases especially in connection with severe haemorrhages of the lungs which must be considered a sufficient cause of death per se However the author has adopted the course according to which all these cases are included under the heading of anoxia because some of

these severe haemorrhages were seen to occur in cases of considerable antepartum bleeding when it cannot be precluded that the child during parturition has inhaled the mothers blood Severe haemorrhages have occurred here in 4 per cent of the cases There might also be some doubt as to the proper classification of some cerebral haemorrhages especially haemorrhages in the cerebral ventricles of which 9 cases are included here in 5 of these as mentioned previously the infants concerned contracted pneumonia The question to arise is wether the pneumonia or the cerebral haemorrhage were the primary cause of death in these cases Furthermore it has been suggested that such haemorrhages in the cerebral ventricles might be due to intrauterine pressure during parturition (14) Haemorrhage in the cerebral ventricles but absence of pneumonia was accompanied by severe haemorrhages of the lungs Babies with pneumonia lived for an average of 9 hours the others for 43 hours all babies belong in the same weight group Otherwise still births are proportionally much more in evidence in the anoxia group viz more than two thirds of the total although still born cases are altogether only about half as numerous as the other cases i e among those brought for autopsy

It is worth noting however that the incidence of anoxia has decreased relatively by more than onefourth cf Table 7 and indeed the same applies to traumatic lesions ($p = 0.0035$) in this field it should be possible to reduce the death rate still more by better hospital service and above all by improved care of expectant mothers this applies specially to mothers living in remote country areas since a large proportion of the most difficult cases which the Obst Dep has to tackle often arrive from remote rural districts at the eleventh hour as virtually hopeless cases

Hyaline Membrane Disease (HMD)

The incidence of HMD is second highest on the list of lethal diseases It is indeed a cause of special anxiety that the relative frequency of deaths has doubled in the last five years of the period in question cf Table 7 $p = 0.003$ and in fact the increase is even more if related to the number of living births in the Obst Dep the latter comparison would be more realistic since this disease affects only infants born alive and more than 90 per cent of the fatalities in this category are brought for autopsy It should be mentioned that this increase is genuine it is not due to the improved diagnostic methods of recent years since all lung samples have now been re examined

If early neonatal deaths alone were taken into account HMD becomes by far the largest single cause of death being responsible in about 26 per cent of the cases According to *Skogstrand & Harnes* (Norway 1960) this is also by far largest cause of death Indeed their figure is 36 per cent which corresponds to the figure found here during

TABLE 7

Perinatal Mortality in Five Year Periods
Percentage of all Autopsy Cases each Period

	1955-59		1960-64	
	cases	per cent	cases	per cent
Congenital malformations	25	14	19	15
Erythroblastosis	6	3	10	8
Anoxia	63	35	32	25
Traumatic lesions	21	12	9	7
Pneumonia	18	10	12	9
Hyaline membrane disease	21	12	31	24
Other known causes	6	3	6	5
Undetermined	20	11	11	8
Total	130		130	

Total births in the Obst. Dep. in the first five year period were 8672 and in the second 6837. Autopsy cases in the group of early neonatal deaths were 105 in the first five year period and 92 in the second.

the second five year period. In this category *Potter* has 21 per cent (USA 1962) and *Butler & Bonham* 18.2 per cent (Gr. Britain 1963).

If we compare the part played by HMD in perinatal mortality here and elsewhere we find a substantially higher rate here, partly because of the large number of still born cases not brought for autopsy.

Most of the children in this group or 70 per cent die within the first 24 hours, see Table 6. This corresponds closely to *Robertson's* findings from Stockholm (1964).

A distinct rise in the number of cases during the period 1952-59 is reported by *Kurtlicz & Downs* (1966) who found the rate of mortality due to HMS to increase from 17 per cent to 24 per cent of perinatal mortality; they found the same tendency towards an increase in later years as that observed in Iceland.

As can be seen from Table 1, perinatal mortality has risen during the last five year period, and a closer study of the table will show that the increase in early neonatal deaths is most marked, viz. from 9.7-10.2 per one thousand, and there is really no alternative but to attribute this increase to HMD.

Congenital Malformations

From a comparison between the two five year periods in Table 7 it may be seen that the relative incidence of congenital malformation is about the same in both periods, or about 14-15 per cent, figures which are similar to those found in the U.S.A. 14 per cent (12), Great Britain 16.6 per cent (1) and Sweden 14.8 per cent (10). The largest number of malformations are found in the nervous system; in this group the proportion of females is higher than that of males.

On the other hand the proportion of males is much higher in this category as a whole or 27 to 17.

Anencephaly is by far the largest individual group of malformations. Autopsies revealed 8 anencephalic cases though two others were known although they were not autopsied since there can be little doubt about this diagnosis the total number of cases can be taken as 10 among 15519 births in the Obst. Dep. during the years 1955–64 making a percentage of 0.065 of all births. This percentage is about the same as that found in Sweden 0.063, Japan 0.063, Greece 0.060 but higher than the figure obtained in Reykjavik for the years 1945–1955 viz. 0.047 (3). The dating and location of cases of anencephaly show that they tend to be grouped in specific years, *e.g.* 3 for each of the years 1955 and 1963 (influenza years). The first two cases in 1955 occurred in the same district and the third case in a family connected with the first case.

This phenomenon is known *inter alia* in England (11).

Pneumonia

The incidence of pneumonia is suspiciously high, 10 percent, with no signs of a change for the better, cf. Table 7. The total number of cases of pneumonia here is proportionally twice that found elsewhere (1, 12) although many pneumonias classed as a secondary cause of death accompanying some other lethal disease were not included, *e.g.* intracranial haemorrhage, as previously mentioned.

Further research is needed here both bacteriological and virological with a view to finding the cause of acute diseases of the lungs. A more extensive and realistic use of antibiotics might be based on the results of such research (2).

SUMMARY

The causes of perinatal deaths were investigated on the basis of 310 autopsies carried out in the Department of Pathology of the University of Iceland in Reykjavik in the years 1955–64.

All these cases were recieved from the Obstetric and Gynaecologic Department of the Landspitali (The University Hospital). During this period 15519 births occurred there, nearly one third of all births in the country for the time concerned. The perinatal mortality rate in the Obst. Department proved to be 31.0 per one thousand, premature births amounting to 5.6 per cent.

The principal causes of perinatal deaths were anoxia 31 per cent, hyaline membrane disease 17 per cent, congenital malformations 14 per cent, pneumonia 10 per cent and intracranial trauma 9 per cent of cases brought for autopsy.

From the first to the second half part of the period under discussion the relative frequency of deaths on account of anoxia and intracranial

trauma fell by nearly 25 per cent In the same period the relative frequency of deaths due to hyaline membrane disease doubled i e from 12 to 24 per cent of cases brought for autopsy

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MITOTIC ACTIVITY OF GASTRIC MUCOSA

A Study by Means of Colcemid

By

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Mitotic activity has been studied in the normal gastric mucosa of rats after injection of colchicine (*Stevens* 1952, *Teir et al* 1952, *Stevens & Leblond* 1953) and regeneration of gastric mucosa in animals after experimentally produced lesions has been studied with histochemical, autoradiographic and ordinary histological methods (*Nicolaysen* 1921, *Hunt* 1958, *Myhre* 1959, 1960, *Townsend* 1961, *Myren & Torgersen* 1964).

In the human gastric mucosa, mitoses and regeneration have been studied by ordinary histological technique (*Heyrovsky* 1913, *Moszkowicz* 1923, *Palmer* 1952, *Teir & Rasanen* 1961) and in one patient by means of tritiated thymidine and autoradiography (*Lipkin et al* 1963).

Topographical studies of mitoses have not been reported previously, and when this study was carried out in 1965, determinations of mitotic activity in the human gastric mucosa by means of kinetostatic substances had not been reported. However, such a study has now been published by *Suzuki* (1966 a, b). The previous studies of mitoses may not give a reliable expression of the cell renewal, viz. mitotic activity, because it is not taken into account that the mitotic count is dependent on mitotic duration (*Evensen* 1962, *Evensen & Iversen* 1962). The longer the mitotic duration, the greater the mitotic count and vice versa, but mitotic rate may still be unchanged. This error is eliminated by the use of a kinetostatic substance which permits the cells to enter mitosis, but effectively blocks the division in metaphase for at least 4–6 hours. By this method the identification of mitoses is also made more easy and safer. The kinetostatic substances colchicine and the related Colcemid (Ciba, Basle), which both are extracted from Colchicum autumnale, fulfil these requirements (*Eigsti & Dustin* 1957, *Evensen* 1962, *Liavåg* 1967).

The aim of the present study was, by means of Colcemid, to 1) register topographically mitoses in the epithelium of stomachs resected for gastric ulcer. The mitotically active part of the epithelium in ulcer

stomachs may differ from the normal pattern Hence 2) the mitotically active compartments of surface epithelium and glands were registered Mitotic activity was related to local pathological processes which were supposed to influence cell division Therefore 3) the distribution and degree of atrophic gastritis and intestinal metaplasia was registered 4) the mitotic counts were determined for glands with intestinal metaplasia and for those without and 5) the mitotic counts were related to the secretion of hydrochloric acid There was no case of gastric carcinoma in the present material but the relation of the findings to development of gastric carcinoma will be discussed

MATERIAL AND METHODS

The material comprises 5 patients with gastric ulcer hospitalized in the Surgical Department II Ullevål Hospital Sex and age distribution of the material is shown in Table 1 From the studies of gastric secretion there is evidence for the distinction of gastric ulcer into three categories 1) Prepyloric ulcer 2) ulcer of the lesser curvature and 3) combined gastric and duodenal ulcers (see *Aagaard* 1963) All the patients in this series had gastric ulcer on the lesser curvature

TABLE 1
Age and Sex of Examined Cases

Patients	Sex	Age in years
1 O T	♂	63
2 A B	♀	54
3 A K	♀	59
4 O K	♂	80
5 M T	♀	69
Mean age		65

The secretion of hydrochloric acid was determined preoperatively by the augmented histamine test (*Kay* 1953)

In experimental animals there is a diurnal mitotic rhythm and the number of mitoses decreases after fasting (*Bullough* 1959 *Leblond & Walker* 1956) hence all the patients were operated on at the same time At 6 30 A M on the day of operation 0 10 mg of Colcemid (Ciba Basle) per kg of body weight was given intravenously With this dose mitoses were arrested in metaphase and only occasional mitoses in anaphase and early telophase were observed The general anaesthesia was started at 9.30 A M and a gastric resection was done The resection was finished in all patients at 10 30 A M and thus the Colcemid time was about 4 hours

The resected stomach was weighed opened along the greater curvature evened out and pinned on corkboard and a photograph was taken It was then immersed in a solution of 10 per cent formalin for 24 hours after which it was removed from the corkboard and a schematic drawing was made of the specimen in ratio 1 1 with description of relevant measures and observations

A 5 mm wide continuous strip was cut from the pyloric to the oral end of the specimens along each curvature The strip from the lesser curvature passed through the ulcer The strips were divided in approximately even blocks the distal end of these was marked with India ink and they were numbered consecutively from the distal part of the lesser curvature (Fig 1) The exact location of each block was marked on the schematic drawing After fixation in 4 per cent formalin and embedding in paraffin a section perpendicular to the mucosa was cut The setting of

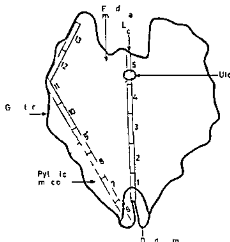


Fig 1

Schematic drawing of specimen from case no 3 A K showing the localization of the examined sections

the microtome was 5 microns The sections were stained with haematoxylin and eosin (H+F) and with PAS and haematoxylin

Mitoses were counted in mucosal squares b means of a graded ocular diaphra m set at 0 16 mm The first square was f cused on a gastric pit and the number of squares from the mucosal surface to muscularis mucosae constituted a mucosal column perpendicular to muscularis mucosae (Fig 2) Each of the histological section were divided into four equally long parts and from each the mitoses were counted in 5 mucosal columns Thus all mitoses were counted in 20 mucosal columns from each histological section In the sections with ulcer the mitoses were counted in 10 columns on each side of the ulcer

In order to compare within one and the same specimen mitotic counts from glands presenting intestinal metaplasia with those in which it was absent about ?500 cells were counted from each of the two types of glands and the number of mitoses among these cells were registered Only the cells in the mitotically active part of the glands were counted in both grou The mitotic counts may differ from area to area hence an equal number of cells were ounted from each of the two gland types within the same section The mitotic counts al o vary from gland to gland and in order to have a basis for comparison glands with the highest mitotic counts were chosen fr m each of the two gland groups

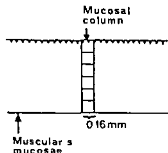


Fig 2

Mitoses were counted in qu r 0 16 × 0 16 mm and th number f squ res from mucosal urface to mu cul s muco e c nstituted a m sal c lunm which as pendicular t muscularis muc a

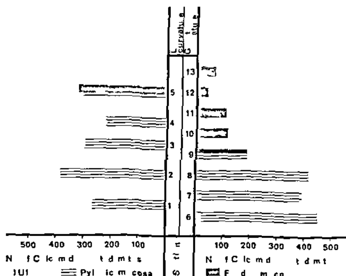


Fig 3
Mean number of colcemid arrested mitoses (mitotic count) related to orientated sections along lesser and greater curvature All specimens

RESULTS

The resected specimens weighed from 125–140 g and the ulcers were either situated in the transitional zone between pyloric and fundic mucosa or within 2 cm from this borderline

With regard to mitotic activity the five specimens fell into two groups *According to this the results of the topographical registration* of mitoses are condensed to show the mean values for the two groups of specimens (Tables 2–5) and for all the five specimens (Fig 3)

Tables 2 and 3 represent a transverse section of gastric mucosa and show the distribution of mitoses in different mucosal levels The mitotic counts given for each histological section is the result of an addition of the number of Colcemid arrested mitoses in parallell squares of the 20 mucosal columns The figures in Table 2 represent the mean values of specimens nos 1 2 and 3 and Table 3 the mean values of specimens nos 4 and 5

The mitotically active part of the glands is longer and the concentration of mitoses is much higher in the pyloric than in the fundic mucosa In the latter the mitoses are localized to the first three or four squares the deeper layers of the fundic mucosa being mitotically inactive The highest mitotic count of fundic mucosa is found in the second square corresponding anatomically to the glandular neck The mitoses of the first square indicate mitotic activity at the bottom of the *gastric* pits

In more than half of the sections with pyloric mucosa mitoses were found in all squares but mitoses were seldom in the first and last

TABLE 2

Mean Mitotic Counts at Different Mucosal Levels Related to Orientated Sections along Lesser and Greater Curvature (Specimens Nos 1, 2 and 3)

Section	Lesser curvature					Greater curvature							
	P				F	P				F			
	1	2	3	4	5	6	7	8	9	10	11	12	13
Mucosal levels	13	11	15	14	13	13	19	18	9	3	2	3	10
	56	43	36	53	44	56	47	60	22	16	23	25	45
	60	33	18	58	43	53	58	46	27	7	9	6	13
	11	34	14	15	21	42	29	17	24	0	1	2	0
	1	13	11	6	21	29	11	9	6	1	0	0	0
		7	7	3	10	19	8	2	1	0	0	0	0
		0	1	0	6	18	2	0	0	0	0	0	0
					0	1	0		0	0	0	0	

Ulcer in this section   P = Pyloric mucosa   F = Fundic mucosa

TABLE 3

Mean Mitotic Counts at Different Mucosal Levels Related to Orientated Sections along Lesser and Greater Curvature (Specimens Nos 4 and 5)

Section	Lesser curvature					Greater curvature					
	P				F	P				F	
	1	2	3	4	5	6	7	8	9	10	11
Mucosal levels	76	53	21	46	73	84	39	24	13	48	46
	162	149	145	126	171	208	182	216	79	82	10
	124	207	221	98	166	171	209	261	93	51	23
	19	173	78	11	63	13	133	159	88	9	2
		35	15	0	0	43	39	5	0	0	0
		10		0	0	8	11	0	0	0	0
		0		0	0		2	0		0	
					0						

Ulcer in this section   P = Pyloric mucosa   F = Fundic mucosa

square corresponding to the superficial part of the gastric pits and to the deep tortuous part of the glands. In the pyloric mucosa the highest mitotic counts are seen in the second or third squares. Anatomically also this corresponds to the neck region of the glands because the gastric pits are deeper in the pyloric than in the fundic mucosa. However high mitotic counts are found even in the fourth square and in several sections from specimens nos 1 and 5 the peak of mitotic activity was found in this square. There are two reasons for this. Firstly the mitoses of ordinary pyloric glands are not only located to the neck region but to the whole straight part of the glands and secondly the pyloric mucosa often contained glands of intestinal type (see Table 8) where the mitoses are located to the depth of the glands.

In Tables 4 and 5 the total mitotic count of 20 mucosal columns the

TABLE 4

Mean Mitotic Counts Related to Orientated Sections along Lesser and Greater Curvature (Specimens Nos. 1, 2 and 3)

	Lesser curvature					Greater curvature							
	P				F	P			F				
Section	1	2	3	4	5	6	7	8	9	10	11	12	13
Total	141	141	102	149	158	231	174	152	89	27	37	36	68
Per column	7 1	7 1	5 1	7 5	7 3	11 6	8 7	7 6	4 5	1 4	1 8	1 8	3 4
Per square	1 8	1 7	1 4	1 9	1 2	2 3	2 0	[illegible]	0 9	0 3	0 3	0 3	0 6

Ulcer in this section P = Pyloric mucosa F = Fundic mucosa

TABLE 5

Mean Mitotic Counts Related to Orientated Sections along Lesser and Greater Curvature (Specimens Nos. 4 and [illegible])

	Lesser curvature					Greater curvature					
	P				F	P				F	
Section	1	2	3	4	5	6	7	8	9	10	11
Total	382	627	479	280	473	648	614	631	273	190	173
Per column	19 4	31 4	24 0	14 0	23 7	32 5	30 8	[illegible]	13 7	9 5	8[illegible]
Per square	5 2	6 5	5 6	3 7	3 8	7 0	6 2	7[illegible]	3 1	[illegible]3	1[illegible]

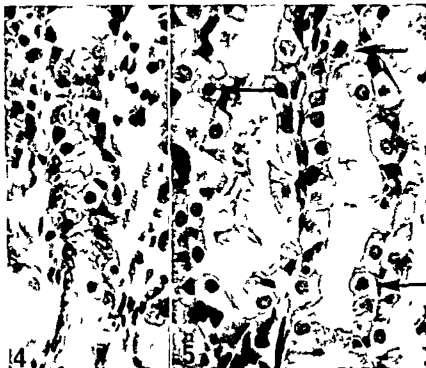
Ulcer in this section P = Pyloric mucosa F = Fundic mucosa

mitotic count per column and per square is shown for each section. The figures are mean values of the specimens within each group. In Fig. 3 the mitotic count of each section represent the mean number of [illegible] arrested mitoses of all the specimens.

The mitotic counts are markedly higher in pyloric than in fundic mucosa. The latter shows smaller variations in mitotic activity from section to section. In pyloric mucosa, however, there is a substantial variation between the sections. From Fig. 3 it appears that there are peaks or plateaus of mitotic activity at several sites in the mucosa. In specimen no. 1 there were two peaks, in no. 3 one peak and in nos. 4 and 5 there were three peaks or plateaus of mitotic activity in each specimen. The sites of highest mitotic activity could differ from specimen to specimen.

In specimen no. 2 the mitotic counts were small, being fairly constant from section to section and showing only slight or inconspicuous differences between pyloric and fundic mucosa. On the other hand the atrophy of pyloric mucosa was greater in this specimen than in the other four. This indicates that there is no parallelism between the degree of atrophy and regeneration.

In the sections with ulcer mitotic activity would be expected to be



Figs 4–5

Fig 4 Characteristic localization of mitoses to mucous neck cells of fundic glands (No 4 O K) × 640 H+E

Fig 5 Mitoses in parietal and chief cell layer (arrows) (No 4 O K) × 640 H+E.

higher than in other comparable parts of mucosa However in Tables 4 and 5 and in Fig 3 it appears that increased regeneration is only present in section 5 while it is rather decreased in section 4 In specimens nos 1 2 and 5 the sections with ulcer showed a mitotic activity which was of about the same magnitude or even lower than that in comparable sections without ulcer In specimens nos 3 and 5 the number of Colcemid arrested mitoses of the sections containing ulcer were two or three times higher than that in other comparable sections Comparable sections contained an equal number of counted mucosal columns from the same type of mucosa

In all specimens mitotic activity was low close to the ulcer but could be normal or above normal at a distance of 2–3 mm from the ulcer

The mitotically active part of the fundic glands is short and the mitoses are usually localized only to the mucous neck cells (Fig 4) However with the Colcemid method it has been found that the parietal and chief cell layer is not mitotically inactive because some mitoses were also seen among the parietal and chief cells (Fig 5) The mitoses were usually localized to the superficial part of the parietal and chief

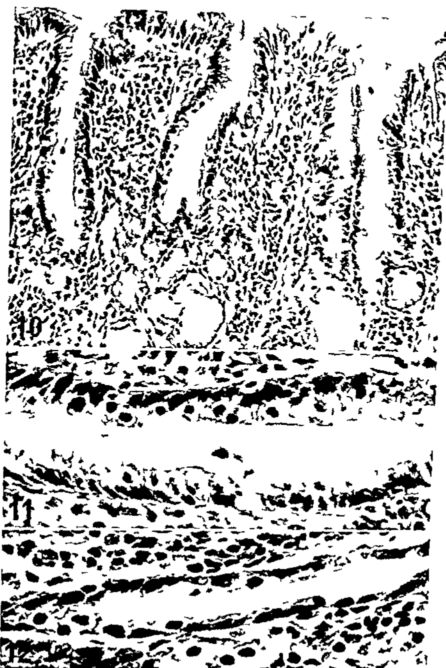




Fig 9
Mitoses in surface epithelium from an area with marked atrophy of pyloric mucosa (No 4 OK) × 640 H+E

Figs 6–8

Fig 6 High mitotic activity of pylori glands Note the length of the mitotically active part (No 4 OK) × 256 H+E
Fig 7 The numerous mitoses in Fig 6 are shown in more detail × 640 H+E
Fig 8 Mitoses in deep tortuous part of pylori gland characterized by clear cells with basally placed disc-shaped nuclei (Direction to mucosal surface left) No 1 OT) × 640 H+E



Figs 10 12

Fig 10 Great number of mitoses in markedly atrophic pyloric glands (No 4 O K) × 256 H+E

Fig 11 One of the atrophic glands in Fig 10 is shown in greater detail × 640 H+E

Fig 12 A gland with flat atrophic epithelium and at least 5 mitoses is penetrating deep into parietal and chief cell layer Direction to mucosal surface left (No 4 O K) × 640 H+E



Fig 13
Markedly atrophic deform gland showing two bud like thickenings of the epithelium consisting of aggregations of active looking cells with three mitoses (arrows) (No 4 O K.) × 640 H+E

cell layer just beneath the neck region of the glands and the cells in mitosis were usually larger than dividing cells elsewhere The findings suggest that division takes place among undifferentiated precursors of parietal and chief cells

In pyloric mucosa the mitoses were most often located to the straight part of the glands from the bottom of the gastric pits to the tortuous part of the glands characterized by pale cells with diskshaped basally placed nuclei However the mitotically active part of pyloric glands may be considerably lengthened showing mitoses well up to the middle of the gastric pit (Figs 6 and 7) and even mitoses in the deep tortuous part of the glands (Fig 8)

In areas with marked atrophy great mitotic activity of the surface epithelium may be found (Fig 9) In atrophic glands the mitotic activity may be considerable (Figs 10 and 11) even where the epithelium



Figs 14–15

Fig 14 High mitotic activity of surface epithelium at the margin of an ulcer (arrow) (No 3 A K) × 640 H+E

Fig 15 Numerous mitoses in intestinal glands of pyloric mucosa (No 4 O K) × 256 H+E

is flat (Fig 12) and buds of active looking cell agregations with mitoses may be found (Fig 13)

Increased mitotic activity was found at the margin of the ulcer in two of the cases (Fig 14)

Two main types of heterotopic intestinal glands were seen in the gastric mucosa In one mitotic activity was of about the same degree as the overall activity of the mucosa The other however revealed signs of intense growth activity *indicated by cells with condensed and basophilic cytoplasm* large hyperchromatic nuclei with prominent nucleoli coarse chromatin dots and numerous mitoses (Figs 15 and 16)

Mitotic activity of Paneth cells has not been reported in the previous investigations According to observations in the present series mitoses

Fig 16

The intestinal glands in Fig 15 are shown in greater detail × 640 H+E



Fig 17

Mitosis in Paneth cell (*arrow*) *Cytoplasm* which in the H+E section was intensely red and granular appears dark (No 1 OT) × 640 H+F

seem to take place also among these cells though it may not be a frequent occurrence (Fig 17)

The limit between pyloric and fundic mucosa is of significance because of their great difference in respect of mitotic activity and development of local pathological processes The ulcers were either located to the transitional zone or to sites within a distance of 2 cm from the latter The extent of pyloric mucosa measured from the pyloric duode

TABLE 6

Extension of Pyloric Mucosa from Pyloro Duodenal Junction along Lesser and Greater Curvature

Patients	Length along lesser curve (cm)	Length along greater curve (cm)
1 OT	8 8	7 2
2 AB	11 0	8 5
3 AK	10 5	8 5
4 OK	12 5	8 5
5 MT	(10 5?)	(13 5)
Mean of 1–4	10 7	8 2

TABLE 7

Distribution and Degree of Atrophic Gastritis Numbers Refer to Orientated Sections along Lesser and Greater Curvature

Atrophic gastritis

Patients	Slight	Moderate	Marked
1 OT	9 10 11 12	1 2 3 5 6 7 8	4§
2 AB	–	11 12 13	1 2 3 4 5§ 6 7 8 9 10
3 AK	10 12 13	11	1 2 3 4 5§ 6 7 8 9
4 OK	–	7 8 9 10 11	1 2 3 4 5§ 6
5 MT	–	5	1 2 3 4§ 6 7 8 9 10

Sections with fundic mucosa § Sections with ulcer
Unmarked ciphers indicate pyloric mucosa

TABLE 8

Distribution and Degree of Intestinal Metaplasia Numbers Refer to Orientated Sections along Lesser and Greater Curvature

Intestinal metaplasia

Patients	Slight	Moderate	Marked
1 OT	4§ 8	1 2 3 6 7	5
2 AB	–	–	–
3 AK	3 6 7	2	1
4 OK	4 9	1 6 7 8	2 3
5 MT	4§	10	1 2 3 5 6 7 8 9

Sections with fundic mucosa § Sections with ulcer
Unmarked ciphers indicate pyloric mucosa

nal junction along the curvatures showed great individual variation (Table 6) and extended up to a higher site on the lesser than on the greater curvature (see also *Landboe Christensen* 1944 *Oi et al* 1959 *Dean & Mason* 1964)

Atrophic gastritis was found in all of the five specimens (Table 7) The changes were most severe in the pyloric mucosa Marked atrophic gastritis was present in all sections containing ulcer

In most cases of intestinal metaplasia the glands contained Paneth cells goblet cells and striated border cells Occasionally only goblet cells and striated border cells were present The metaplasia was mostly located to the pyloric mucosa (Table 8) where atrophy also was most severe Glands of intestinal type were only encountered in two sections from the fundic mucosa (Table 8) Metaplasia seems always to be associated with chronic gastritis (*Graham & Schade* 1965) However marked atrophic gastritis may be present without coexistent meta plasia Thus in specimen no 2 intestinal metaplasia was absent though atrophy was diffuse and marked Metaplasia was not found in close vicinity of the ulcers Only in the specimens nos 1 and 5 slight intes tinal metaplasia was present in both ends of the ulcer containing sec- tions at a distance from the ulcer

TABLE 9

Mitotic Count of Pyloric Mucosa Showing Intestinal Metaplasia Compared with Mitotic Count of Pyloric Mucosa without Metaplasia within same Specimen and Sections

Pyloric mucosa

Patients	Intestinal metaplasia			Without metaplasia			X	P
	Cell count	*Mit* oses	*Per* cent	*Cell* count	*Mit* oses	*Per* cent		
1 O T	2466	301	12 21	2470	244	9 88	7 04	< 0 01
2 A B	–	–	–	(2411)	(184)	(7 63)	–	–
3 A K	2461	345	14 02	2455	264	10 75	11 93	< 0 001
4 O K	2910	634	21 79	2514	482	19 17	5 36	< 0 05
5 M T	2498	394	15 77	2548	306	12 01	15 02	< 0 001
Total	10335	1674	16 20	9987	1296	12 98	32 29	< 0 001

The above figures denoting mitotic activity were obtained from randomly chosen glands including metaplastic as well as non meta plastic glands In pyloric mucosa the mitotic activity was examined separately for the two types of glands A corresponding study of the fundic mucosa was not possibl because intestinal metaplasia was restricted to the pyloric mucosa only In all specimens with intestinal metaplasia the mitotic activity of the heterotopic intestinal glands was significantly higher than that in ordinary pyloric glands without meta plasia (Table 9)

The relationship between the mitotic activity of the pyloric mucosa and the secretion of hydrochloric acid is shown in Table 10. In cases 1 and 4 the secretion was within normal range while the remaining three patients were hypoacid. Case 2 had the lowest output of hydrochloric acid and also the lowest mitotic count. Microscopically the resected specimen showed marked atrophy of the pyloric mucosa but absence of intestinal metaplasia. In case 5 the figure denoting acid secretion was nearly as low as in case 2 but the mitotic count was the second highest in the series. Microscopically, however, there was great difference between specimens 2 and 5. Although both showed marked atrophy there was marked intestinal metaplasia throughout the whole pyloric mucosa in case 5. Case 4 had the highest output of hydrochloric acid and also the highest mitotic count. Thus, according to findings in this small series there is apparently no correlation between the mitotic count of pyloric mucosa and the secretion of hydrochloric acid. Marked mitotic activity may be found in stomachs with normal secretion or with hypoacidity.

TABLE 10

Mitotic Count of Pyloric Mucosa Related to Output of Free Hydrochloric Acid after Augmented Histamine Test

Patients	Per cent of Cells in mitosis	Free HCl in mEq/hr
1 O T	11.04	13.81
2 A B	7.63	4.64
3 A K	12.39	9.11
4 O K	20.58	20.66
5 M T	13.87	6.7[illegible]
Mean	*13.10*	*11.00*

DISCUSSION

Teir & Rasanen (1961) found in non diseased portions of gastric mucosa that the number of mitoses was significantly higher in pyloric than in fundic glands. In both types of glands the mitoses were located to the so called mucous neck cells. They did not find mitoses in parietal, chief or pyloric cells and mitoses in surface epithelium was rare. In experimentally produced lesions of the gastric mucosa in rats *Hunt* (1958) and *Townsend* (1961) found greatly increased mitotic activity of the surface epithelium at the rim of the lesion until the gap was covered. As evidenced by the present findings a similar increase in regenerative activity also seems to take place in the human gastric mucosa accompanying conditions with increased cells loss as ulcers and atrophy.

The consensus seems to be that parietal and chief cells are stable and that these cells do not regenerate in man. By a histochemical method *Myren & Torgersen* (1964) did not find any evidence of regeneration of parietal cells in mice following experimentally produced lesions of

gastric mucosa *Hunt* (1958) and *Townsend* (1961) however who used an ordinary histological technique found that experimentally induced lesions of the gastric mucosa in rats healed with complete differentiation of the glands *Myhre* (1960) found by means of tritiated thymidine and autoradiography of intact fundic mucosa in rats that labelled cells were not only located to the isthmus and neck of the glands but a few labelled cells were even observed in the depths of the fundic glands From these observations *Myhre* concluded that also parietal and chief cells seemed to be able to divide and that regeneration of these cells to some extent took place in the depths of the fundic glands This is in agreement with observations in the present study

Teir & Rasanen (1961) found significantly higher mitotic counts in gastric mucosa from patients with gastric ulcer (and cancer) than in gastric mucosa from patients with duodenal ulcer On the condition that the mitotic duration is the same in the two groups of patients the finding indicates a more rapid turnover and/or a failing differentiation of the epithelium in ulcer stomachs The difference in cellular proliferation and differentiation may be due to a higher frequency of pathological processes such as cancer development atrophic gastritis and intestinal metaplasia in stomachs with gastric ulcer than in those with duodenal ulcer (*Heyrovsky* 1913 *Magnus* 1954 *Larson et al* 1961) Atrophic gastritis may influence the turnover time of the epithelium and intestinal metaplasia indicates a disturbance in cellular differentiation *Nicolaysen* (1921) and *Konjetzny* (1947) found that gastritis always accompanied gastric ulcer and in this series atrophic gastritis was present in all of the resected specimens The mucosal atrophy indicates increased loss of cells and homeostasis is only achieved by increase in mitotic activity *Heyrovsky* (1913) and *Moszkowicz* (1923) found increased regeneration of gastric epithelium in ulcer stomachs In the present series increased mitotic activity was found at the margin of two gastric ulcers and in atrophic mucosa a lengthening of the mitotically active part of the glands was demonstrated indicated by mitoses in the surface epithelium and in the depth of the glands

Teir & Rasanen (1961) reported that the mitotic count was lower in glands with intestinal metaplasia than in those without but these authors did not use a kinetostatic substance and it is hardly justified to assume that the mitotic duration in the two types of cells is identical In the present investigation there was a significantly higher mitotic activity in glands with intestinal metaplasia than in those without The result is in agreement with findings in rats after colchicine had been injected The daily renewal of ileal epithelium was 70 5 per cent while the corresponding figures for the epithelium of pyloric and fundic mucosa was 54 and 25 4 per cent respectively (*Leblond & Stevens* 1948 *Stevens* 1952 *Stevens & Leblond* 1953 *Teir & Rasanen* 1961)

Crile (1958) has advanced the theory that the growth of a cell type

is controlled by the interaction between growth inhibiting and growth promoting compounds the former being a tissue specific signal substance produced by the cell itself the latter a specific trophic hormone produced by some other type of cell The incidence of gastric carcinoma is increased in patients with pernicious anaemia (see *Siurala & Seppala* 1960 *Schindler* 1965) and about 70 per cent of the patients with gastric carcinoma have little or no free hydrochloric acid *Crile*, therefore asks whether achlorhydria might result in a prolonged stimulation of the antral mucosa because the negative feed back is lacking and whether the fundic mucosa in turn might be constantly stimulated by the antral hormone gastrin If so an increasing mitotic activity of the pyloric mucosa with decreasing output of hydrochloric acid might be expected Such a correlation however was not found but a larger material is necessary if the question is to be settled

It might be relevant to ask whether mitotic activity and development of carcinoma in the stomach might be interrelated It is well known (*Nicolaysen* 1921 *Morson* 1955 b) that the pyloric region and the lesser curvature i e that part of the stomach which is covered by antral mucosa is the site of predilection for a development of carcinoma In the present series pyloric mucosa showed significantly higher mitotic activity than the fundic mucosa

Already in 1842 *Cruveilhier* claimed that carcinoma may develop in the margin of a chronic benign gastric ulcer in patients predisposed to cancer (see *Aagaard* 1963) This supposition has later been evidenced by many investigators (*Hauser* 1883 *Konjetzny* 1913 1938 *Magnus* 1954 *Larson et al* 1961 *Priestley* 1962 *Oota* 1963) *Magnus* (1954) found unequivocal ulcero cancer in 1 9 per cent among 421 patients operated on for gastric ulcer In the present investigation 2 out of the 5 examined specimens showed increased mitotic activity in the sections containing ulcer compared with that in the surrounding mucosa

In the same specimen there were often several peaks of mitotic activity which could be located to the ulcer margin and/or to sites at a distance from the ulcer Similarly *Bamforth* (1956) found in five patients operated on for gastric ulcer that the early carcinomatous changes could be multifocal they could be located to the ulcer margin or to sites at some distance from the ulcer

Several investigators claim that intestinal metaplasia is involved in gastric carcinogenesis and thus may be regarded as a precancerous lesion (*Jarvi & Lauren* 1951 *Morson* 1955a b *Graham & Schade* 1965) Thus *Jarvi & Lauren* (1951) found striated border cells in 50 per cent and intestinal type mucus in 30 per cent of a series of 184 carcinomas and 6 papillomas of the stomach In the present investigation a significantly higher mitotic activity was found in glands with intestinal metaplasia than in comparable glands without metaplasia

In conclusion there seems to be a correlation between mitotic ac

tivity and development of carcinoma in the stomach When areas with high mitotic activity seem to be sites of predilection for the development of carcinoma the explanation might be that the growth control is more easily disturbed by carcinogenic agents in cell populations with high mitotic activity than in those where such activity is low

SUMMARY

By means of the Colcemid method the mitotic activity was studied in the mucosa from 5 stomachs resected for gastric ulcer

The mitotic counts were significantly higher in the pyloric than in the fundic mucosa The mitoses were more closely packed and the mitotically active part was longer in the pyloric than in the fundic glands

In the fundic mucosa the mitoses were almost restricted to the bottom of the gastric pits and the glandular necks However at deeper sites in the mucosa some mitoses were also found among the parietal and chief cells indicating a slow regeneration even of these cells

In the pyloric mucosa mitoses were usually located to the straight part of the glands from the bottom of the gastric pits to the tortuous part of the glands

In pyloric and fundic glands the highest mitotic counts were usually located to the neck region of the glands In areas of intestinal metaplasia however the highest mitotic counts were at deeper sites in the mucosa because in intestinal glands the mitoses are located to the lower part of the crypts

In atrophic mucosa there was often evidence of increased regeneration indicated by lengthening of the mitotically active part of the epithelium with mitoses both in the surface epithelium and in the deep part of the glands Buds of active looking cell aggregations with mitoses were seen in atrophic glands with otherwise flat inert looking epithelium

In two of the five specimens there was a greater mitotic activity in the sections containing ulcer than in the surrounding mucosa In the remaining three specimens the sections containing ulcer showed a mitotic activity of about the same magnitude or even lower than that in the surrounding mucosa

The mitotic counts especially of the pyloric mucosa often varied considerably from section to section resulting in several peaks or plateaus of mitotic activity which could be located to the margin of the ulcer and/or far from the ulcer

A type of heterotopic intestinal glands is described which is characterized by cells with basophilic cytoplasm and large hyperchromatic nuclei In all specimens with intestinal metaplasia these glands showed a significantly higher mitotic activity than the mitotically most active

non metaplastic glands Mitoses were sometimes seen among cells reminding of Paneth cells

No absolute correlation between the mitotic activity of the pyloric mucosa and the maximum output of free hydrochloric acid was found

Areas with highest mitotic activity seem to be sites of predilection for a development of carcinoma

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A *Parietal Mesothelium*

Immediately following the injection of tracer substance no thorium dioxide was observed in the mesothelium (Fig 1) In ½–2 minutes a few particles were detected on the surface of the microvilli (Fig 2) At 2–3 minutes more particles were present on the microvilli and on other parts of the surface membrane (Fig 3) At 5 and 10 minutes evidence of a substantial absorption was observed (Figs 4–11) Thorium particles were present on microvilli (Figs 4 6 and 7) along the surface membrane (Figs 4 and 6) inside pinocytic vesicles (Figs 4 5 and 6) and apparently free in the cytoplasm (Figs 6 and 10) Frequently tracer particles were identified in invaginations of the surface membrane (Figs 4 and 5)

At 15 30 and 60 minutes following application most thorium in the cytoplasm was present inside electron dense bodies (Figs 12–18) These were bordered by a single triple layered membrane and measured from 0 3–0 8 μ in diameter Their content of tracer substance seemed to increase during the first hour (Figs 12 and 17) Thorium containing dense bodies were present also after 2 3 and 4 hours but their number was decreasing with the lapse of time Frequently dense bodies and mitochondria were lying in close proximity (Figs 13 14 16 and 18)

Tracer particles were not observed along the membranes of the endoplasmic reticulum or inside the mitochondria Golgi apparatus and nuclei The intercellular spaces with tight junctions and desmosomes appeared normal (Figs 3 10 and 11) In a few instances particles were observed immediately above or below an intercellular space (Figs 12 and 13) They were however never detected inside the space itself

From 5 minutes and onwards tracer particles were observed along the basement membrane sometimes in the subcellular space between the plasma membrane and the basement membrane Frequently they were lying close to the opening of cytoplasmic vacuoles (Figs 11 12

Legends to Figs A–D

Pericardial mesothelium following the injection of the commercial Thorotrast solution Varying degrees of cell damage with unsatisfactory preservation of cytoplasmic organelles (see text)

Fig A 10 minutes following injection Some particles are scattered in the cytoplasm while others appear to have been engulfed in a vacuole (V) Parietal surface × 18 000

Fig B 30 minutes after injection Thorotrast particles are concentrated in electron dense bodies (DB) The cytoplasm is vacuolized (V) and pseudopodal foldings (P) of the surface membrane are present Mitochondrium (M) Parietal surface × 45 000

Fig C 1 hour Concentrations of Thorotrast in mesothelium (MES) and in the submesothelial area (arrows) The localization of particle aggregations is indistinct Parietal surface × 18 000

Fig D 1 hour Thorotrast-containing multivesicular bodies (see text) is present between collagen fibres (COL) Parietal surface × 45 000

and 13) Particles were present also in the submesothelial tissue underneath the basement membrane (Figs 4 6 8 and 10) which sometimes appeared irregular or absent (Figs 9 and 11)

B Visceral Mesothelium

During the first 15 minutes following injection of thorium dioxide no particles were encountered (Fig 21) After 20 and 30 minutes they were present in small amounts on microvilli, on other parts of the surface membrane and inside the cytoplasm (Figs 22 and 23) As a rule they were observed inside vesicles and vacuoles (Figs 24 and 26) At 30 and 60 minutes some dense bodies containing tracer substance were found (Fig 2a) Their appearance seemed identical with the dense bodies in the parietal mesothelium but the number of thorium containing bodies was far less in the visceral cells At 2 3 and 4 hours thorium containing dense bodies were only occasionally detected

The intercellular spaces were devoid of tracer substance The basement membrane appeared continuous in most sections

Particles were only observed in the cytoplasm of vacuole containing mesothelial cells The LR cells were not seen to contain tracer substance (Figs 27–31) However their abundant endoplasmic reticulum and numerous dark ribosomes sometimes made it difficult to exclude the presence of thorium dioxide particles

C The Submesothelial Tissue

On the parietal side free thorium particles were present in the submesothelial tissue from 5 minutes following application (Figs 1 and 10) and onwards They were lying between bundles of collagen (Figs 4 8 and 12) separately or in small clusters At 15 and 30 minutes tracer substance was observed also in the cytoplasm of macrophages which occurred in large numbers At 60 minutes thorium particles were present in nearly all macrophages observed Occasionally particles appeared free in their cytoplasm but most of these were concentrated in vacuoles and particularly in electron dense bodies (Figs 17 18 and 19) These bodies appeared similar to the dense bodies observed in mesothelial cells (Figs 16 and 17) At 2 3 and 4 hours thorium containing macrophages were still present in the submesothelial tissue but in decreasing numbers (Fig 20)

On the visceral side some thorium containing macrophages were observed after 2 and 3 hours Their number however was far lower there than in the parietal membrane

Fibroblasts lymphocytes and granulocytes were also present in the submesothelial area of both surfaces (Figs 3 11 15 and 20) None of these cells contained tracer substance Few blood capillaries were detected They were never seen to contain tracer particles in their lumen or cytoplasm



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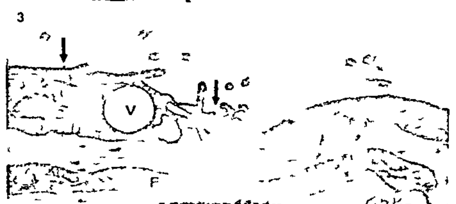
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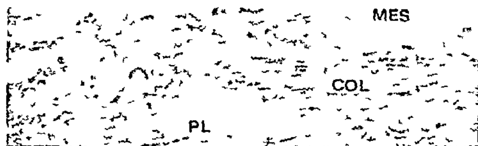
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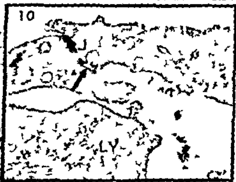
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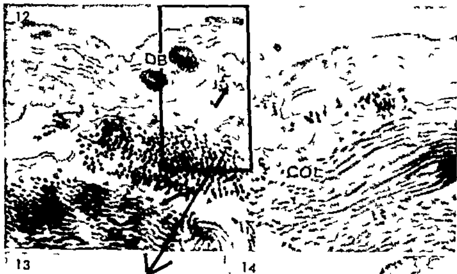




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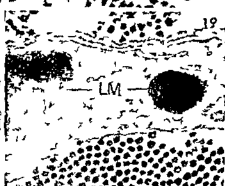
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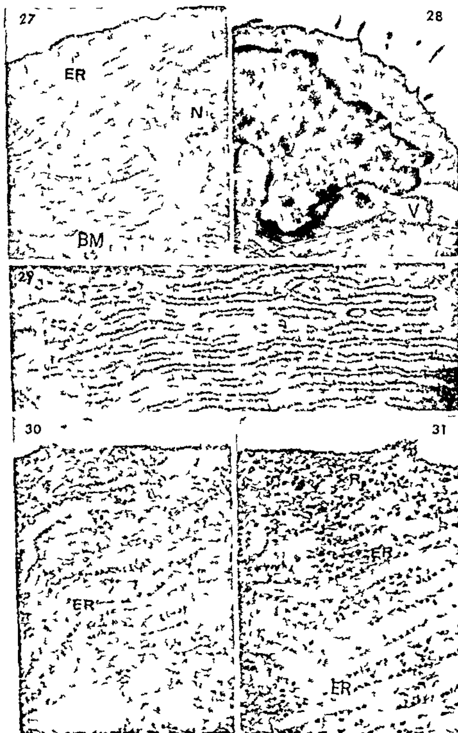
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cytoplasmic structure when Thorotrast (7 22), iron saccharate (14 20) and other colloidal substances (19 22 23) are applied to cell surfaces This has been explained by Sinapius (19) as blasige Quellung due to heavy accumulations of hydrophilic colloid in the cytoplasm

In the present study changes indicating acute cell damage followed the employment of the commercial Thorotrast preparation Vacuolization of cytoplasm and microvilli (13), pseudopodal folding of the surface membrane (24) and general loss of ultrastructure were observed Corresponding alterations occurred in mesothelium exposed to the suspension medium alone

When the suspension of washed particles was injected however the ultrastructure of the mesothelial cells appeared normal Since all other steps in the three series of experiments were identical it is suggested that washing of the particles removes the injurious effects which presumably are caused by the suspension medium The thorium dioxide particles *per se* do not seem to produce acute cell damage as evaluated from the ultrastructure

Absorption of thorium dioxide was found 15–20 minutes earlier in the parietal than in the visceral mesothelium When the first few particles were observed in the visceral mesothelium the absorption through the parietal membrane appeared to be at its maximum (Figs 12–17 and Fig 22) At all stages the concentration of thorium particles was much greater in the parietal than in the visceral membrane

Systematic comparisons between the absorption from the visceral and parietal surfaces of body cavities are very few As far as the authors are aware electron microscopic studies of this kind have not been reported Investigations of the pleural cavity employing other techniques indicate that a fluid turnover takes place *Stewart & Burgen* (21) measuring fluid movements into and out of the pleural space concluded that a passage of fluid from pulmonary capillaries takes place through the visceral pleura into the cavity and out through the parietal pleura *Thorsrud* (25) injecting Imferon into the pleural cavity observed iron deposits far earlier in the parietal than in the visceral mesothelium

In the present study tracer particles were observed also in the visceral pericardium although in far smaller amounts than on the parietal side Theoretically this observation might reflect a re entry of particles absorbed from the parietal pericardium with secondary deposits in visceral lining cells Several facts however speak against this theory

a) The mechanism of absorption appeared identical on both surfaces with initial adhesion of particles to the surface membrane
b) Particles were not observed within capillaries in the subepicardial tissue
c) Thorium dioxide is transported via the lymphatics and stored in the reticulo endothelial system (6 7) Hence a re-entry through

the general circulation within a time interval of 20 minutes is considered very unlikely

It is concluded from the present study that absorption of thorium dioxide takes place from both pericardial surfaces but mainly through the parietal mesothelium The delay and disparity between absorption from the two surfaces indicate the presence of a fluid turnover in the pericardial cavity with the main fluid movement directed towards the parietal membrane

The mechanisms involved in the absorption seemed identical in the vacuole containing cells of both surfaces Initially particles were seen adhering to microvilli and surface membrane The extensive participation of microvilli in the process of absorption supports the common theory (16 23) that their main function is to increase the absorptive area

Free particles appeared in the cytoplasm and in the submesothelial tissue during the early stages of absorption It is unlikely that this observation reflects knife carry artefacts First the tissue was sectioned partly from the luminal partly from the contraluminal surface without apparent influence on the amount and localization of free particles Second the free particles disappeared from the mesothelial cells with the lapse of time If their presence were due to knife carrying one would expect free particles to be present at all time intervals

It is a matter of controversy whether particles can travel through the cytoplasm as free particles without a carrier substance Ferritin seems to traverse cells without any encircling membrane in mesothelium (4 20) and in endothelium (4 14) Thorotrast also is reported to travel in this manner (3 4) an observation which is supported by the present study

From 5 to 30 minutes following application tracer substance was observed in vesicles and vacuoles some of which appeared to be in communication with the pericardial lumen or with the subcellular space These observations are in accordance with the results of previous studies on Thorotrast (4 6 7 22) colloidal gold (17 20) and lipids (22 23)

With the lapse of time thorium containing vesicles were rarely encountered It has previously been shown in studies on lymphatic endothelium (3 4) that material lying free or in small vesicles rapidly left the cells while substance in larger vacuoles was retained for considerably longer periods of time

From 15 minutes and onwards the main concentration of thorium dioxide occurred inside electron dense cytoplasmic bodies Accumulations of tracer substance were present within some of these even 4 hours following application

Electron dense bodies are commonly present in pericardial mesothelial cells (11) The ultrastructure of these elements seems to cor

respond with the concept of lysosomes as classified by previous authors (8) In the present study many of the dense bodies were seen to contain thorium dioxide particles The morphological characteristics of the dense bodies with and without tracer particles appeared identical Accordingly it may be questioned whether these bodies should be regarded as lysosomes or as phagosomes (8 15)

In any event it is suggested that the dense bodies do belong to the heterogenous group of cytoplasmic organelles comprising lysosomes phagosomes residual bodies a o It is also suggested that these dense bodies play an important part in the mesothelial absorption of thorium dioxide

In the present study a small number of vacuoles were seen to contain tracer particles together with a less electron dense amorphous material (Fig 26) Whether this observation reflects an intermediate stage between pinocytosis and lysosome activity cannot be answered on the basis of the present investigation

In experiments employing the commercial Thorotrast preparation accumulations of tracer substance were observed as large aggregates or vacuoles Their membrane when identifiable appeared similar to the limiting membrane of the dense bodies described in this and in a previous study (11) The Thorotrast containing vacuoles and multi vesicular bodies described by other authors (6 7) also show striking similarities with the large vacuoles encountered in the present study (Figs B and C) It is considered probable that many of the vacuoles or vesicular *bodies reported by previous authors are in fact lysosomes or* phagosomes which have been altered by the Thorotrast suspension medium and/or the fixation procedure

Any conclusions can not be drawn as regards the way in which the thorium particles traverse the basement membrane In the proximity of the thorium accumulations the basement membrane sometimes appeared irregular or absent (Figs 13 and 16) but this was not the rule Several free particles were observed in the subcellular space but they were *not detected while actually traversing the basement mem* brane Large fenestrations of the membrane like those in the diaphragmatic mesothelium (1 10) were not observed Heavy accumulations of tracer particles along the basement membrane suggesting a barrier function (17) were not encountered

The thorium dioxide particles were observed only in the ordinary vacuole containing mesothelial cells No absorption seemed to take place in the scattered visceral cells containing an abundant endoplasmic reticulum (Figs 27–31) *This observation is in support of* the view that the ER containing cells are not macrophages (11)

Thorium particles were never observed in the intercellular spaces although sometimes they were present close to their luminal or basal terminations In studies on lymphatic endothelium Casley Smith (3 4) *occasionally observed Thorotrast particles in the intercellular spaces*

If the lymphatics were rendered abnormally permeable (5) the cell junctions were supposed to act as inlet valves Similar mechanisms were never encountered in the present investigation no matter whether commercial Thorotrast or washed particles were employed

In the submesothelial tissue free particles were observed during all stages of absorption With the lapse of time they also accumulated in macrophages mainly inside electron dense bodies with similar characteristics as the dense bodies encountered in the mesothelium The size of these bodies however appeared more variable in the macrophages than in the mesothelial cells Their ultrastructural appearance suggests that they belong in the group of lysosomes/phagosomes and seem to play an important part in the phagocytosis of thorium dioxide

The other cells present in the submesothelial tissue did not appear to participate in the absorption and transport of thorium dioxide particles

SUMMARY

Pericardial absorption was studied at intervals of from 30 seconds to 4 hours following injection of Thorotrast or of washed thorium dioxide particles into the pericardial cavity of rats

The quantity of absorption seemed to take place through the parietal pericardium A smaller amount of particles was observed also in the visceral mesothelium Particles were present 15–20 minutes earlier in the parietal than in the visceral membrane indicating a movement of fluid across the pericardial space towards the parietal pericardium

The tracer substance was absorbed through the mesothelial cells as free particles in pinocytic vesicles and vacuoles and in membrane bounded dense bodies interpreted as lysosome like structures

Only the vacuole containing mesothelial cells were seen to absorb tracer particles The scattered ER containing cells on the visceral surface did not participate in the absorption Tracer particles were never observed in the intercellular spaces In the submesothelial tissue thorium dioxide was observed in the form of free particles and inside the cytoplasm of macrophages mainly concentrated in lysosome like dense bodies

The observations also suggest that acute cell damage from Thorotrast is caused by the suspension medium and not by the thorium dioxide particles

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PERICARDIAL ABSORPTION OF THORIUM DIOXIDE IN RATS

2 *A Lymphangiographic Study*

By

TROND KLUGE and AKSEL A ONGRE

Received 20 viii 67

The importance of the lymphatic system for the drainage of serous cavities is well established (5 18 24) Several investigations have been carried out on the lymph drainage from the peritoneum while studies on pericardial lymphatics are few The need for more information on the latter subject is stressed by several authors (14 23 24)

Previous works on the anatomy of cardiac and pericardial lymphatics have been based upon dye injections with subsequent autopsy studies (1 8 15) Lymphangiography does not seem to have been employed in studies on pericardial lymph drainage This method however has the advantage of allowing *in vivo* examinations as performed in the present work

Colloidal thorium dioxide (Thorotrast) was chosen as a tracer substance for this investigation It is absorbed and transported almost exclusively by the lymphatic vessels and has been used in studies on lymph drainage from the peritoneal cavity (5 12 13 16) and in studies on the thoracic duct (5) At the same time Thorotrast is a convenient tracer for electron microscopy and radioactive analyses thus permitting a combination of methods

The purpose of the present investigation was as follows

1 To visualize the main lymphatic pathways from the pericardium
2 To obtain information concerning the velocity and direction of absorption and drainage

The nomenclature in the present work refers to the anatomical studies by *Job* (8) and *Miotti* (15)

MATERIALS AND METHODS

23 adult male rats weighing 300–350 g were used in the study. They were operated upon in endotracheal anaesthesia with ether/alcohol (10). Thoracotomy was performed on the left or right side alternately and 0.3 cc of Thorotrast (Fellows Testagar Co., Detroit, Michigan, USA) was introduced into the pericardial cavity. The lung was re-expanded and the chest and tracheostomy closed. The animals were radiographed immediately following injection of Thorotrast while lying on the operating table. Consecutive radiographs were taken at time intervals from 30 min up to 48 hours and after 16 days. Prior to each examination the animals were anaesthetized with ether alcohol (2:1) on an open mask. Between examinations they were awake and were allowed free access to water and a standard diet.

Supplementary Examinations

In five animals the thoracic duct was catheterized in the neck according to methods described by Saldeen & Linder (19). Dissection was performed after principles outlined by *Engeset* (6). To visualize the topography of the duct Thorotrast was injected in a retrograde direction.

In two animals simultaneous injections were made into the pericardium and the right pleura with the purpose of visualizing the right pleural and diaphragmatic lymph drainage.

In two animals 2 cc of contrast medium was injected to outline the borders of the pericardial cavity. In two animals 5 cc were injected. This resulted in rupture of the pericardium.

In one animal Thorotrast was instilled into the oesophagus 6 hours after pericardial injection.

Radiographic Technique

All animals were examined on the Siemens Elinograph using the *serial spot film* device. Agfa Gevaert Curix RP film was used in casettes provided with Siemens Universal Saphir intensifying screens. Focus size was 1.2 mm, focus film distance about 70 cm, object film distance about 50 cm, giving a magnification of 1.4.

*Exposure values were KV 40–mAs 16. The animals were positioned under fluoro*scopic guidance in postero-anterior, lateral and oblique views. This method gave a rapid and easy performance with reliable positioning of the animals.

Six out of the 23 animals were examined postmortally lying on envelope packed Agfa Gevaert Osray DW non screen film, *the films placed on lead rubber to obviate* backscatter. Focus size was 1 mm, focus film distance 32 cm and exposure values KV 40–mAs 50. This technique gave a superior image quality and is recommended where high definition is desired in the radiographic image.

Alle films were developed by hand.

RESULTS

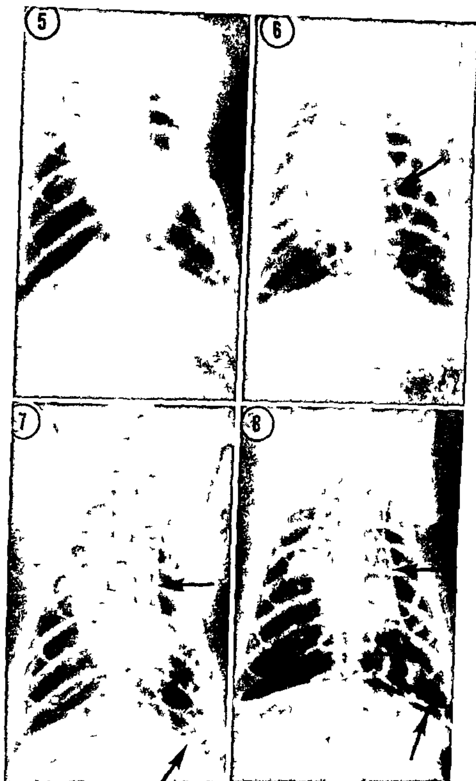
Immediately following injection the heart shadow appeared with slightly increased density (Figs. 2, 3 and 4) owing to the spread of contrast medium within the pericardial cavity. The first lymphatics were visualized 1½–2 hours following injection (Fig. 6) and opacification of the different pathways increased during the first 10–14 hours (Figs. 7–13). From 16–24 hours the density of contrast in the lymphatics appeared almost constant (Figs. 14–16). At 34 and 48 hours the opacification was decreasing (*Figs. 17 and 19*) and *after 16 days* the lymph vessels were no longer visible (Fig. 20). Accumulation of tracer substance in regional lymph nodes increased from 14 hours onwards (Figs. 13, 15 and 19) and was marked even *after 16 days* (*Fig. 20*).


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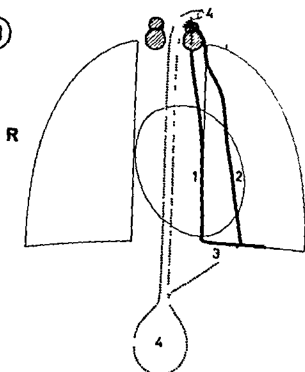

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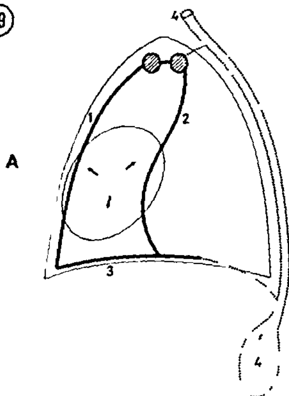



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Legends to Figs 1-29

Figs 1 Postero anterior view prior to injection of Thorotrast

Figs 2 and 3 Anteroposterior and lateral views immediately following injection Mediastinum and heart shifted to the left Slightly increased density of heart shadow owing to distribution of contrast medium within the pericardial cavity

Fig 4 Lateral view ½ hour following injection Lymph vessels still not visible

Fig 5 Postero anterior view 1 hour Slight opacification along left heart border

Fig 6 Slightly oblique projection 2 hours Opacification of left mediastinal lymphatics (arrow)

Figs 7 and 8 Postero anterior view 3 hours Increasing density of left mediastinal and diaphragmatic lymphatics (arrows)

Fig 9 Lateral view 4 hours Long tortuous diaphragmatic lymph vessel is visible (arrow)

Fig 10 Lateral view 6 hours Arrow indicates communication beteween mediastinal and diaphragmatic lymph vessels

Figs 11 and 12 Oblique projections 8 and 10 hours Increasing opacification of parasternal (upper arrow) and mediastinal (lower arrow) lymphatics

Fig 13 Lateral view 14 hours Lymph nodes in upper mediastinum (upper arrow) collect contrast medium from parasternal and mediastinal (middle arrow) lymphatics Communications between the two systems are present Lower arrow indicates lymph vessels of diaphragm

Fig 14 Oblique view 16 hours Anastomosis between mediastinal and diaphragmatic lymphatics is visualized (arrow)

Fig 15 Lateral view 20 hours Animal hanging in supine position horizontal beam Parasternal lymphatics (vertical arrow) draining towards upper mediastinal lymph nodes (horizontal arrow)

Fig 16 Slightly oblique projection 25 hours Still marked opacification of the three main pathways (see text)

Fig 17 Lateral view 34 hours Several communications between the different systems of lymph drainage

Figs 18 and 19 Postero anterior and lateral views 48 hours Contrast medium in lymph vessels (arrow) is decreasing Accumulation of Thorotrast in lymph nodes

Fig 20 Lateral view 16 days Contrast medium in lymph nodes (arrows)

Fig 21 Postero anterior view immediately following injection of 2 cc of tracer solution The pericardium appears distended

Fig 22 Postero anterior view 8 hours after injection into pericardium and right pleura Left and right parasternal lymphatics are visible (arrows) Opacification also of diaphragmatic lymphatics with drainage caudally towards cisternal lymph nodes

Fig 23 Antero posterior view 8 hours after injection of large volume (5 cc) The pericardium has ruptured Thorotrast is accumulated in spleen

Fig 24 Retrograde injection of Thorotrast through catheter (upper arrow) in thoracic duct Lower arrow indicates level of possible anastomoses from diaphragmatic lymphatics

Fig 25 Same animal *in vitro* radiograph Middle arrow indicates level of possible anastomoses from mediastinal and parasternal lymphatics Leakage of contrast medium from lower portion of duct and cisterna chyli (lower arrow)

Fig 26 Caudal position of catheter with the tip above the diaphragm Visualization of caudal segments of thoracic duct with cisterna chyli (arrow)

Fig 27 6 hours after injection Demonstration of parasternal (1) mediastinal (2) and diaphragmatic (3) lymphatics The relationship to the posterior mediastinum outlined by contrast medium in oesophagus (arrow)

Figs 28 and 29 Schematic drawings of the main pathways in pericardial lymph drainage Anteroposterior and lateral views 1 Parasternal lymphatics 2 Mediastinal lymphatics 3 Diaphragmatic lymphatics 4 Thoracic duct (visualized only by direct injection) Levels of possible anastomoses with the thoracic duct are indicated by dotted lines

The following pathways were identified

1 The left *parasternal* lymphatic appearing as a main trunk in the anterior mediastinum (Figs 11 and 15) It was highly opacified in all animals and followed a course towards the upper mediastinal lymph nodes (Figs 13 and 15) After simultaneous injection into the pericardium and the right pleura both parasternal lymphatics were visualized (Fig 22)

2 The *mediastinal* lymphatics usually forming 1–2 larger vessels in the middle portion of the mediastinum (Figs 7 12 and 14) These vessels also appeared to drain into the upper mediastinal lymph nodes (Fig 13) Frequently communications with the lymph vessels of the diaphragm were demonstrable (Figs 10 and 14)

3 *Diaphragmatic* lymphatics were also visible in all animals Generally they appeared with high density forming a plexus of variable pattern Commonly the larger vessels were running dorsally (Figs 9 10 and 13) and communicating with mediastinal lymphatics (Fig 14) After simultaneous injection into the pericardium and the right pleura the diaphragmatic vessels were visualized in a greater number Their pattern suggested drainage also caudally towards cisternal lymph nodes (Fig 22) The thoracic duct was not opacified following pericardial injection of Thorotrast The duct was visualized by retrograde injection through a catheter (Figs 24 25 and 26) Anastomoses from the above pathways to the thoracic duct were suspected but could not be demonstrated with certainty in the radiographs The anatomical relations between the lymphatic pathways and other mediastinal structures were determined by instillation of Thorotrast into the oesophagus (Fig 27)

A volume of 0 3 cc was not sufficient to outline the borders of the pericardial cavity (Figs 1–4) These were visualized by injection of a larger volume (Fig 21) however a certain degree of cardiac tamponade occurred Injection of 5 cc led to rupture of the pericardium (Fig 23)

The main pathways of lymph drainage as outlined in this study are presented in schematic drawings (Figs 28 and 29)

DISCUSSION

The present investigation confirms that Thorotrast is a convenient radiopaque substance for studies on lymph drainage (5 16) The employed method allowed studies to be performed *in vivo* which is considered superior to methods simulating heart beat in dead animals (4)

The assumption set forth by *Drinker et al* (2 3) that only electrolytes are absorbed by the pericardial lymphatics is not confirmed by the present study On the contrary our results support the conclusion that colloidal substances are easily absorbed from body cavities (22)

In the present work a small volume of 0 3 cc was injected under

direct vision to ensure that distention of the pericardial sac was minimal This intention was probably fulfilled as the extraluminal heart shadow (20) did not appear widened following the injection of tracer substance

At the same time 0 3 cc was a sufficient volume for the demonstration of the main lymphatic pathways If a larger volume had been applied other routes or communications might perhaps have been visualized However as pointed out by *Wilson et al* (23) large volumes frequently produce cardiac tamponade or even pericardial rupture (Fig 23)

The investigation demonstrated drainage of Thorotrast through 3 main pathways the *parasternal mediastinal* and *diaphragmatic* lymphatics Several communications were detected between these systems mainly in the upper and lower areas of the mediastinum It should be considered however that drainage may have taken place also via other pathways where the intravasal concentrations of Thorotrast were too low to permit radiographic visualization Minor individual variations in the lymphatic pattern were occasionally detected The general picture however was in agreement with anatomical studies in rats (8 15) and was not influenced by these variations

The thoracic duct was not opacified in any period following pericardial injection This is in accordance with other studies (5 16) stating that even the peritoneum and diaphragm are drained preferably along pathways other than the thoracic duct and particularly via the parasternal lymphatics

When the thoracic duct was visualized by direct injection (Figs 24–26) no retrograde filling of the abovementioned pathways occurred Anastomoses between the duct and the mediastinal lymph nodes were occasionally supected but they were never obvious

The lympho venous communications in the neck seem to vary in different animal species *Ffskind* (5) demonstrated in experiments on rabbits that the parasternal lymphatics emptied separately into the veins of the neck Only in very rare instances did drainage take place through communications with the upper segment of the thoracic duct It is reported that the parasternal and mediastinal lymphatics in rats drain into the upper segment of the thoracic duct mainly through the upper mediastinal lymph nodes (15)

No conclusions can be drawn from the present study as to the presence of such communications The interposition of lymph nodes may well explain that no retrograde filling of parasternal lymphatics was obtained following injection into the thoracic duct *Olin & Saldeen* (16) conclude from studies with Thorotrast that a relatively small amount of diaphragmatic lymph in rats is drained through the thoracic duct This observation however is dependent on the position of the draining catheter in the thoracic duct If the parasternal and mediastinal lymphatics empty into the duct in its cranial segment a more distally posi

tioned catheter will occlude this pathway and only the drainage from the more caudal segments will be recorded. The relative importance of the thoracic duct in pericardial lymph drainage will be discussed in a later publication.

Peripheral lympho-venous anastomoses (11) were not detected in the present study. Visualization of such communications could not be expected considering the volumes and techniques employed. Some authors (7, 15) claim that such communications are artificial, produced by the injection pressure.

Lymphangiographic studies on pericardial drainage have not, to our knowledge, been reported. In an autopsy study on mammalian hearts (17) it was concluded that pericardial drainage takes place through mediastinal lymphatics to the mediastinal lymph nodes. *Allen* (1) reported pericardial drainage of dyes from the base of the heart into the pretracheal and left tracheal lymph nodes in dogs. In the present work the observations indicate that lymph drainage from the pericardium takes place also in other directions, i.e. anteriorly towards parasternal lymphatics and caudally to diaphragmatic lymphatics. These results seem to support observations from electron microscopic studies (9) that a large proportion of injected thorium dioxide is absorbed through the parietal pericardium. It should be added that the concentrations and volumes of tracer substance employed in these studies are identical.

Anatomical studies on rat lymphatics (15) suggest that the lymphatics of the heart proper forms two main trunks from the base of the heart draining into the mediastinal lymphatics and never through the parasternal system. If a substantial portion of the injected substance were absorbed from the visceral pericardium into epicardial lymphatics, an opacification of these trunks and their communications with the larger mediastinal lymph vessels might be expected. However they were not visualized in the present study, thus speaking against a heavy absorption of Thorotrast from the visceral pericardium.

The main lymph vessels were visible at 1½–2 hours following Thorotrast injection, and maximum opacification occurred from 6 to 16 hours later. *Olin & Saldeen* (16) reported the opacification of diaphragmatic lymphatics at the end of 1 hour when Thorotrast was injected into the peritoneal cavity. Experiments with tagged erythrocytes (18, 22, 23) also suggest that absorption from the pleura and peritoneum is more rapid than that from the pericardium. According to a report on experiments with Imferon injected into the pleural cavity, *Thorsrud* (21) found a prompt increase of serum iron, the maximum being attained after 24 hours. The absorption of colloidal iron seemed to take place through the parietal pleura.

It is to be expected that the results will differ with the injected substance, concentration and volume, and probably with mechanical factors (position of the animal, heart beat, respiratory movements). A comparison between the rate of absorption from different body cavities

lies beyond the scope of this investigation It is suggested however that X ray studies with radiopaque tracer substances might yield additional information in this respect

SUMMARY

X ray examinations were performed on living rats after injection of colloidal thorium dioxide into the pericardial cavity Radiographs were taken at time intervals from 30 minutes up to 16 days The investigation suggests that pericardial lymph drainage takes place along 3 main pathways, i e the left parasternal mediastinal and diaphragmatic lymphatics Anastomoses between these systems were detected

Other routes of pericardial lymph drainage were not demonstrated Communications to the thoracic duct were not visualized However the possibility that such communications may exist could not be excluded

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ENDOTRACHEAL ANAESTHESIA FOR INTRATHORACIC SURGERY IN RATS

By

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Intrathoracic surgical procedures are completely dependent upon reliable methods of anaesthesia

Most respirators designed for anaesthesia in small animals are expensive complex and imply the use of mechanical or electrical devices (2 4 7 8) Simpler systems as suggested by *Lumb* (6) and *Siegler* (9) do not offer facilities for controlled ventilation In rats difficulties connected with tracheostomy have been an obstacle to endotracheal anaesthesia (3)

A simple method for anaesthesia in rats submitted to thoracotomy is described in the present paper The system combines ideas obtained from anaesthesia in human subjects and in animals and is based upon intermittent positive pressure ventilation through a tracheostomy

APPARATUS

A diagram of the equipment is given in Fig 1 Oxygen flows through a simple vaporizer containing ether/alcohol (2 1) The mixture of oxygen and anaesthetic is *directed into the trachea through a polyethylene catheter inserted into the* tracheostomy By passing of oxygen may be obtained by an extra tubing and a 3 way valve An opening of 0 5 × 1 cm is made in the rubber tube between the valve and the tracheostomy catheter (Figs 1 and 2)

PROCEDURE

Anaesthesia is induced by open mask administration of ether/alcohol (2 1) Tracheostomy is performed by a 2 mm horizontal incision between the 2 and 3 tracheal ring A polyethylene catheter (O D 1 8 2 mm) is introduced and connected with the tubing system (Fig 2) Oxygen flow is checked on the flowmeter (Fig 1) prior to the thoracotomy We have utilized a flow of 150–200 ml/min

Artificial ventilation is achieved by intermittent closure of the hole in the tubing Inflation of the lungs is easily controlled (Figs 3 and 4)

For prolonged procedures p rticularly in the posterior mediastinum it may become necessary to deflate the lung completel in order to get access to the structures in question (Fig 5) In the e instances the c llapsed lung should be expanded every 2–3 minutes to avoid d elopment of atelectases

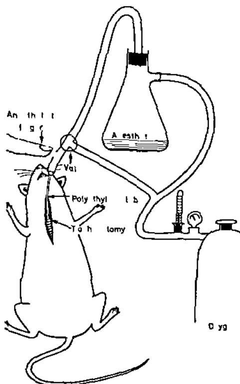


Fig 1
Diagram of ventilation system The anaesthetist s finger is intermittently closing the hole in the tubing whereby inflation and deflation of the lungs is obtained

At termination of the procedure it is ensured that the lung is completely expanded before the wound in the chest wall is closed *The tracheostomy is closed* immediately after withdrawal of the endotracheal tube For this purpose we have employed a single non traumatic suture of 6–0 nylon which is passed around the tracheal ring above and below the incision

DISCUSSION

A *Ventilation system* As will be seen from the diagram (Fig 1) the design is simple and inexpensive It may be constructed in a few minutes *and has no movable parts Breakdown of the arrangement for ventilation* and administration of anaesthesia has not occurred In our experiments respiratory volumes and resistance have not been recorded These parameters however may be recorded by means of additional equipment (1)

In our opinion the described arrangement is easier to adjust and handle than the more complex mechanical and electrical devices Variations in respiration and anaesthetic level can be corrected by manual adjustments and can be undertaken immediately and whenever necessary

The method requires an extra assistant to take care of the ventilation



Figs 2–3

Fig 2 Animal during thoracic surgery The polyethylene catheter (C) is inserted through the tracheostomy Connection with the tubing is obtained by a small cannula and the distal portion of a syringe (S) The finger is withdrawn from the hole (H) and the left lung is collapsed The retractor (R) is made from a safety pin

Fig 3 Higher magnification of the operative field The left lung (L) is inflated The anterior portion of the pericardium (P) is visible The retractor separates the wound edges with ribs (R) and muscles (M)



Figs 4–5

Fig 4 The lung (L) is partly deflated Easy access to the anterior mediastinum *with the heart (H) covered by the pericardium Tracer substance is injected into the pericardial cavity Ribs (R) Thoracic muscles (M)*

Fig 5 Large left thoracotomy The lung (L) is completely deflated in order to get access to the posterior mediastinum with the aorta (A) and oesophagus (O) A ligature is placed around the aorta

procedure It may be considered a drawback that the surgeon cannot both do the surgery and control the anaesthesia On the other hand it is an advantage that the anaesthetist will have one hand free for assistance in the operative field

B *Tracheostomy* The employment of a tracheostomy is in our opinion superior to oral intubation (5) and to devices where a mask is made to fit tightly over the head of the animal (6 9) Previous authors report on difficulties concerning the techniques of tracheostomy intubation and closure *Farris & Griffith* (3) recommend excision of a 2 × 2 mm piece of the anterior tracheal wall They state that intubation through a tracheostomy is suitable only for acute experiments as the tracheostomy wound cannot be closed This view is not supported by our experience When a horizontal incision of approximately 2 mm is made between two tracheal rings intubation is easy and the subsequent closure can be made without difficulties A single suture has been found sufficient for complete closure without leakage or deleterious tension It has not been necessary to inhibit the secretion by means of atropine

Due to the relatively long distance from the thyroid cartilage to the tracheal bifurcation the catheter may be introduced several cm into the tracheal lumen Accordingly some sliding of the catheter may be allowed without interference with the ventilation Leakage around the tube does not occur if the tracheal incision is of the proper size

C *Area of application* The present method of anaesthesia has been employed in more than 200 procedures on the heart pericardium (Fig 4) and thoracic aorta (Fig 5) On several occasions bilateral thoracotomies have been performed as well as repeated intrathoracic procedures on one and the same animal Frequently the surgery has lasted for more than 1 hour Complications due to the method of anaesthesia have not occurred

The described system has been safe simple and reliable for intrathoracic procedures in rats Most probably it will prove to be useful in other small laboratory animals

SUMMARY

A method for endotracheal anaesthesia in rats is described The system is simple and reliable utilizing oxygen and a mixture of ether and alcohol No movable or electrical parts are included The performance is easy and risks of mechanical breakdown are minimal

Details are given concerning the techniques of tracheostomy intubation and closure of the tracheal incision

The method has been employed in more than 200 intrathoracic procedures without complications

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RENIN LOCATION IN THE DIFFERENT PARTS OF THE JUXTAGLOMERULAR APPARATUS IN THE CAT KIDNEY

2 *Fractions of the Afferent Arteriole, The Cell Group of Goormaghtigh, the Efferent Arteriole and the Glomerulus*

By

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Received 13 vi 67

In a previous investigation (*Faarup* 1967) the renin content of the macula densa and of the granulated cells in the juxtaglomerular part of the afferent arteriole was investigated More than 95 per cent of the renin activity in the two fractions was found to be localized in the af ferent arteriole In the present paper other fractions of the juxtaglome rular apparatus were studied the efferent arteriole and the cell group of Goormaghtigh as well as different parts of the preglomerular vascular system and the glomerulus

MATERIAL AND METHODS

25 micron thick freeze dried sections from Neutral Red stained kidneys from cats nos 6 9 15 and 16 (the preparation of which has been described in the preceeding paper) were used The microdissection was identical with that previously used with freeze dried sections with the exception of the isolation of the glomeeruli which was more easily done by hand using a fragment of razor blade held in an artery forceps The further treatment of the tissue fractions was identical with the fore going In estimating the renin content in the cell group of Goormaghtigh the cell number from the serial sections (*Faarup* 1967) was used as a basis for compari son The quantity of renin found in the afferent arteriole from the same juxta glomerular apparatus was also measured. Here only subcapsular nephrons were used for microdissection

For the investigation of the renin content of the interlobular arteery of fractions of the afferent arteriole and of the efferent arteriole the length of the vessel in cluded in the assay was used as a basis of compa son The number of cells with and without juxtaglomerular granules was also counted in the individual fractions (Table 3)

The afferent arteriole was divided nto three fractions (Fig 2) 1) the proximal region close to the interlobular artery 2) the inte medi ry part of the arteriole and 3) the juxtaglomerular distal part of the c s l Afferent arterioles we e selected

in which juxtaglomerular granules were seen at the branching point from the inter lobular artery and in which granulated cells were absent from the intermediary region (Fig. 2). Such preparations as well as preparations of glomeruli and of efferent arterioles were taken from the outer third of the renal cortex.

To identify the efferent arteriole in the juxtaglomerular apparatus it was essential to first determine the position of the afferent arteriole close to the glomerulus (Fig. 3).

Renin estimation in the microdissected tissue was based upon incubation of the tissue extracts with angiotensinogen as previously described (Faarup 1964).

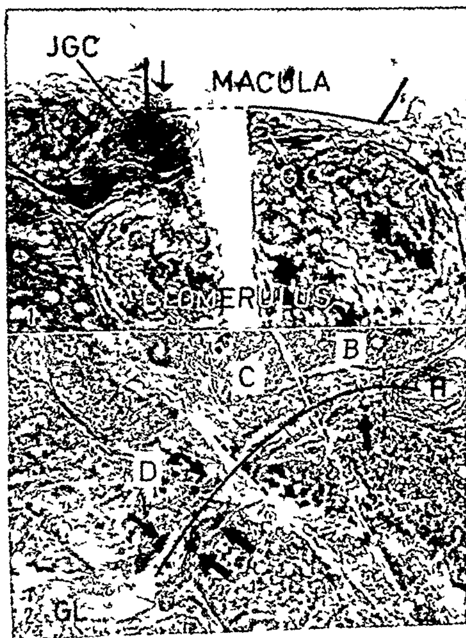




Fig 3

The efferent arteriole (EA) has been separated from the glomerulus (GL) (cut tangentially in the section) as well as from the afferent arteriole in which several epithelioid cells with juxtaglomerular granules (JGC) are seen The macula densa is not included in this section DT distal tubule (Freeze dried section vitally stained with Neutral Red × ??0)

Figs 1–2

Fig 1 In the juxtaglomerular appar tu the c ll gr u[illegible] f G ormaghtigh (GG) h s been separated from the affe nt arteri le [illegible]nta ning g anulated epithe lioid cells (JGC) a [illegible]ell a from the ma ula den a [illegible] h [illegible]h as previously removed A f v ma ul ells ar till pre ent i the va [illegible] l r l acti n (arrow) Freeze drie l section vitall t ined w th Neutral Re l × [illegible]0

Fig 2 In the afferent art [illegible] le the lum n I indi ated l the ur d line extending from its or g n t the inte l l ila arter (A) to the gl merulus (GL) The arteriol s pa te l in three p rt The proximal f act on close to the interlobul arte [illegible] B) th nt rme liat fra t on (C) and the distal fraction near the gl m rulus (D In the rteriole lls ntain ng juxtaglomerular gra ule are found at th [illegible] and ne r th gl m rulus (arrows) The sections hi l s parate the d ff rent fra tions re m ked l v the dotted lines The i terlobula artery was later rem ved fr m fra t on B (× 160)

in which juxtaglomerular granules were seen at the branching point from the interlobular artery and in which granulated cells were absent from the intermediary region (Fig 2) Such preparations as well as preparations of glomeruli and of efferent arterioles were taken from the outer third of the renal cortex

To identify the efferent arteriole in the juxtaglomerular apparatus it was essential to first determine the position of the afferent arteriole close to the glomerulus (Fig 3)

Renin estimation in the microdissected tissue was based upon incubation of the tissue extracts with angiotensinogen as previously described (*Faarup* 1967)

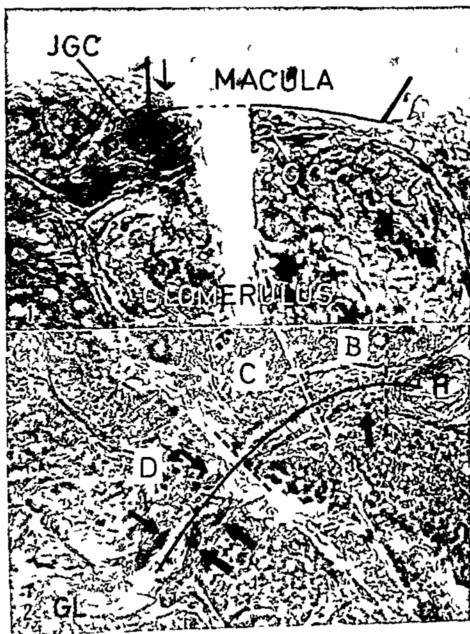


TABLE 2

Renin Content in Different Fractions of the Vessels from the Interlobular Artery to the Efferent Arteriole (cf Fig 4)

	Cat no	Total length of the vascular fraction in micron	No of ramifications without granulated cells	No of granulated cells	Renin content in (U × 10^{-4} per 100 microns length of the vessel
A	9	4800	20	0	0 01
Interlobular artery	15	2180	13	0	0 03
Mean values		3490	17	0	0 02
B			No of ungranulated cells		
Afferent arteriole (proximal part)	9	370	152	19	0 7
	9	445	72	14	0 4
	9	200	25	13	1 8
	15	225	40	9	0 2
	15	315	31	9	0 8
Mean values		317	64	13	0 7
C	9	635	118	0	0 2
Afferent arteriole	9	1340	209	0	<0 1
(middle part)	9	680	118	0	0 3
	15	990	193	0	<0 1
	15	630	90	1	0 1
Mean values		855	146	0	0 1
D	9	600	32	46	4 2
Afferent arteriole	15	260	9	32	6 2
(distal part)	9	260	42	23	7 2
	15	135	6	12	8 6
	15	385	12	21	7 1
	15	230	11	24	4 8
Mean values		320	19	26	5 9
E	9	335	24	9	0 4
Efferent arteriole	9	230	36	4	0 3
(with granulated cells)	15	205	27	5	0 2
Mean values		255	29	6	0 3
F	9	470	2	0	<0 1
Efferent arteriole	15	280	30	0	≤0 1
(without granulated cells)					
Mean values		375	28	0	<0 1

In each fraction the number of granulated and ungranulated cells in the vessel was counted and the vascular length was measured. In the last column the renin content per 100 microns in length of the vessel is given. For each group of fractions tested, the mean value was calculated. It is seen from the table that changes in renin content per 100 microns of vascular length parallel the number of granulated cells in both the preglomerular part of the vascular system and the efferent arteriole

D) *In the distal part of the afferent arteriole*, with a large number of cells containing numerous juxtaglomerular granules the renin content per 100 *microns* of vascular length was 4.2×10^{-4}–8.6×10^{-4} GU with a mean value of 5.9×10^{-4} GU (Table 2 D)

Γ) *In the efferent arteriole containing* cells with a small number of *juxtaglomerular granules*, the renin content per 100 *microns of vascular* length was 0.2×10^{-4}–0.4×10^{-4} GU with a mean value of 0.3×10^{-4} GU (Table 2 E)

F In two preparations of *efferent arteriole devoid of granulated cells* 0.1×10^{-4} GU per 100 microns of vascular length or less were found (Table 2 F)

Glomerulus with or without the Place of Entrance of the Afferent Arteriole

It is seen in Table 3 that the content of renin was below the limits of sensitivity of the method in preparations of glomeruli in which no granulated cells occurred (less than 0.05×10^{-4} GU per glomerulus) preparations in which granulated cells were found contained small amounts of renin

TABLE 3

Renin Content of Neutral Red Stained Free e Dried Sections of Glomeruli with and without the Site of Entrance of the Afferent Arteriole Renin Was only Found in the Former in Which Cells Containing Juxtaglomerular Granules Were also Seen

	Glomeruli in which the site of entrance of the afferent arteriole was present		Glomeruli in which the site of entrance of the afferent arteriole was *not* present	
Cat no	6	9	6	9
No of isolated sections of glomeruli	23	25	45	46
Glomerular volume in microns³ × 10⁶	16.3	18.0	38.9	39.8
No of granulated cells	7	5	0	0
Renin content in GU × 10⁴	0.7	1.3	<0.5	<0.5

DISCUSSION

It appears from Table 1 that *the amount of renin in the cell group of* Goormaghtigh is very low if at all present In various fractions of the vascular system of the nephron from the interlobular artery *to the* efferent arteriole the renin content was significantly higher in fractions in which cells containing juxtaglomerular granules were present than in those where such cells were absent (Table 2 and 3 Fig 4) This is in

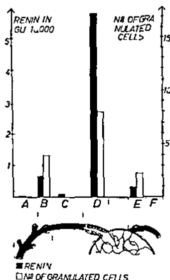


Fig 4

Renin content of different fractions of the vascular system of the nephron correlated to the occurrence of epithelioid cells containing juxtaglomerular granules The renin content and the number of granulated cells are given as mean values peer 100 microns length of the vascular fraction (cf Table 2) In vascular segments in which no juxtaglomerular granulees were found (the interlobular artery and some of its ramifications (A) the intermediate part of the afferent arteriole (C) and most efferent arterioles (F)) very little or no renin was present contrary to the findings in the fractions containing granulated cells (some proximal segments of the afferent arteriole (B) the distal part of the afferent arteriole (D) and some efferent arterioles (F)) In addition it is seen that the renin content per granulated cell was found to be higher juxtaglomerularly in the afferent arteriole than elsewhere in the arterioles

accordance with publications of *Bing & Wiberg* 1958 *Bing & Kazimierczak* 1959 and *Cook* 1960 in which the interlobular artery and the glomerulus were found to be without measurable quantities of renin Fig 4 shows that the content of renin varies with the content of cells containing juxtaglomerular granules in the vessels However the amount of renin per granulated cell was higher in the juxtaglomerular part of the afferent arteriole than in granulated cells located elsewhere This finding is in accordance with the higher amount of granules in the single granulated cell of the distal part of the afferent arteriole

Based upon the results presented in this and in the preceding paper (*Faarup* 1967) the renin distribution in the juxtaglomerular apparatus can be depicted as shown in Fig 5 from which it appears that the renin content of the afferent arteriole contributes by more than 90 per cent of the total amount found in the juxtaglomerular apparatus

As the investigation by *Bing & Kazimierczak* (1964) and *Endes et al* (1965) on kidneys of newborn animals had shown that quite a high amount of renin could be demonstrated in fractions of renal cortex in

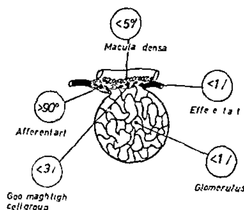


Fig. 5

Renin distribution in the different fractions of the juxtaglomerular apparatus and glomerulus in freeze dried tissue

which only immature nephrons (lacking cells with juxtaglomerular granules) were present the conclusion of the present investigation should be limited to the location of renin in the different parts of the juxtaglomerular apparatus in the normal fully developed cat kidney *In such cases location of renin is related to the presence of granulated* epithelioid cells in the vascular system the main part of the renin being localized in the juxtaglomerular region of the afferent arteriole

On the basis of experiments on the rat in which pieces of the renal cortex were autotransplanted *Gomba* (1967) has reached essentially the same conclusions Similarly *Hartroft et al* (1961 1963 1964) using fluorescent antirenin were able to localize renin only to the granulated cells of the juxtaglomerular apparatus

SUMMARY

In a previous study of the location of renin in the cat kidney (*Faarup* 1967) the renin content of the macula densa—if present—was found to be very small In continuation the quantity of renin found in the inter lobular artery fractions of the afferent arteriole the cell group of Goormaghtigh the glomerulus and the efferent arteriole were studied in microdissected freeze dried cryostat sections vitally stained with Neutral Red

A fairly good correlation was found between the occurrence of cells containing juxtaglomerular granules (some of the proximal parts of the afferent arteriole the distal part of the afferent arteriole and some of the efferent arterioles) and the amount of renin present the main part being located in the distal part of the afferent arteriole The renin found in the cell group of Goormaghtigh and the glomerulus amounted to less than 3 per cent and 1 per cent respectively of the content of the afferent arteriole

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A MEMBRANE FILTER METHOD FOR THE DEMONSTRATION OF BACTERIA BY THE FLUORESCENT ANTIBODY TECHNIQUE

3 *The Application of the Method for the Demonstration of Enteropathogenic* Escherichia coli *in Drinking Water*

By

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A suitable method by which to use non fluorescent membrane filters (MF) for immunofluorescent identification of bacteria in a water sample trapped on such a filter has been described in a previous paper (4) The method allowed a quantitative determination of the bacteria identified but its sensitivity was limited in bacterial concentrations below 1 000 bacteria per litre This drawback was overcome with the use of an enrichment procedure and in laboratory experiments it was possible to make an identification of bacteria within 4–6 hours when they were present in concentrations of 2–50 organisms per litre of water (5)

The present paper presents the results from the application of the fluorescent antibody (FA) technique combined with the MF technique for a rapid demonstration of enteropathogenic Escherichia coli (EEC) in accidentally contaminated drinking water in two communities in Sweden In one of the communities the contaminated drinking water caused an outbreak of gastroenteritis probably due to EEC

MATERIAL

The supplies of drinking water in two communities of Middle Sweden were accidentally contaminated during the summer and autumn of 1965 Water samples were examined for the coliform density and also for the occurrence of EEC. The background of this is shortly outlined below

Community I An outbreak of gastroenteritis occurred in the summer of 1965 in two residential areas (Bergsbrunna and Vilan) in Uppsala Sweden Four hundred and forty two individuals fell ill within a period of 14 days with a concentration of 261 sick persons on 28th–30th July Bacteriological examinations of faecal specimens showed the occurrence of EEC. During the course of the epidemic three serotypes of EEC, 026 B6 0111 B4 and 0128 B12 were isolated Examinations for

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Salmonellae and Shigellae as well as virological examinations gave negative results The epidemic, which has been fully described elsewhere (?) was suspected to be waterborne owing to its explosive character This suspicion was supported by the fact that the supply of drinking water was in common for both Bergsbrunna and Vilan and a bacteriological examination of the drinking water revealed high numbers of coliform bacteria Water samples were collected from the municipal supply of drinking water and examined for coliform density and for FEC as described under Methods

Community II This community called Gimo is situated 50 kilometers north of Uppsala It has one new and one old drinking water supply A small river (the Oland river) runs close to the new water supply Early in October 1965 after a period of heavy raining a bacteriological examination showed that the new drinking water supply was polluted with coliforms Water samples were then collected both from the old and from the new water supplies from the Oland river and also from a well nearby They were all examined for the coliform density and also for the occurrence of EEC as described under Methods

From the epidemiological point of view it should be noted that a few cases of acute gastroenteritis occurred during the time when the drinking water was polluted Unfortunately faecal specimens from these patients were not examined bacteriologically When it became known that the new drinking water supply was polluted sufficient chlorination of it was rapidly instituted and after no coliforms could be demonstrated in the water and no more cases of gastroenteritis occurred

METHODS

Estimation of Coliform Density

The standard multitube method (MPN method) was used for the estimati n of the coliform density (1)

MF Procedures

Water samples of 1 litre were filtered through black non fluorescent MF (Millipore HAB(P)G 047) and through ordinary white MF (Millipore HAW(P)C 047) From the black non fluorescent MF circular pieces 12 5 mm in diameter were stamped out with a metal die and subsequently stained with FA as described elsewhere (4) Each of two or three white MF were put into tubes containing 10 ml of nutrient broth and incubated at 37 C for enrichment After 4 and 6 or 14 hours the broth was filtered through black non fluorescent MF (Millipore HAB(P)G 025) and subsequently stained with FA This technique is called the two step p ocedure and has been fully described before (5) The broth from the remaining tube was centrifuged after enrichment for 14 hours Ordinary smears were prepared from the sediment and subsequently stained with FA in the usual way

FA Procedures and Fluorescence Microscopy

Sera for the different serotypes of EFC were obtained from rabbits immunized according to the directions of *Edwards & Ewing* (7) The following serotypes were used 026 B6 055 B5 086 B7 0111 B4 0112ab B13 0112ac B14 0114 B? 0119 B14 0125 B15 0126 B16 0127 B8 and 0128 B12 The globulin portion of each antiserum was conjugated with fluorescein isothiocyanate (11) or lissamine rhodamine B (10) as described elsewhere (3)

FA staining of ordinary smears and n n fluorescent MF as well as fluorescence mi roscopy were performed in th s desc ibed before (4 5)

Conventional Culture and Serological Procedur s

Conradi Drigalski s blue agar plates were inoculated with b oth in which ordinary white MF had been nriched for 14 hour Aft incubation at 37 C for 20–24 hours about 40–50 coloni s were tested with l de agglutination tests to determine the K antigen The O ant gen was confirm d by the current tube agglutination technique after the growth of positive colon es in b oth and boil ng at 100 C for 90 m nutes (7)

TABLE 1

Bacteriological Examination of the Supply of Drinking Water in Community I (Bergsbrunna & Vilan)

Coliform density per litre water 35 C	Coliform density per litre water 44 C	Serotypes of EEC identified with the MF technique in combination with the FA technique: Direct FA staining of non fluoresc MF	Enrichment (6 hrs) and FA staining of non fluoresc MF	Enrichment (14 hrs) and FA staining of ordinary smears	Serotypes of EEC isolated by conventional culture and serology
9 000	3 000	0111 B4	026 B6 0111 B4 0128 B12	026 B6 0111 B4 0128 B12	026 B6 0111 B4 0128 B12

See Methods

TABLE 2

Bacteriological Examination of River Water and the Supply of Drinking Water in Community II (Gimo)

Source of water	Coliform density per litre water 35 C	Coliform density per litre water 44 C	Serotypes of EEC identified with the MF technique in combination with the FA technique: Direct FA staining of non fluoresc MF	Enrichment (14 hrs) and FA staining of non fluoresc MF	Enrichment (14 hrs) and FA staining of ordinary smears	Serotypes of EEC isolated by conventional culture and serology
1) The new water supply	800	300	Neg	026 B6 0114 B?	026 B6 0114 B?	0114 B?
2) The old water supply	80	10	Neg	Neg	Neg	Neg
3) A nearby well	900	300	Neg	0125 B15	0125 B15	0125 B15
4) The Oland river	90 000	6 000	Not done	0125 B15	0125 B15	0125 B15

See Methods

RESULTS

Bacteriological Examination of Drinking Water in Community I (Bergsbrunna & Vilan)

The results of the bacteriological examination of the water supply for Bergsbrunna and Vilan are presented in Table 1

It will be seen that the drinking water contained 9 000 thermolabile and 3 000 thermostable coliform organisms per litre EEC 0111 B4 was demonstrated on FA stained non fluorescent MF through which 1 litre of water had been filtered Counts of the FA stained organisms revealed that they occurred in concentrations of 3–5 per 10 fields of vision This corresponds to a concentration of about 1 000 organisms per litre (4) FEC 026 B6 0111 B4 and 0128 B12 were demonstrated on FA stained non fluorescent MF through which broth enriched for 6 hours (see Methods) had been filtered (the two step procedure) The FA stained bacteria occurred rather abundant on the filters Negative results were obtained with broth enriched for 4 hours The 3 serotypes of EEC were also demonstrated on FA stained ordinary smears prepared from broth in which white MF had been enriched for 14 hours

The same serotypes of FEC demonstrated with the MF technique combined with the FA method were isolated from faecal specimens of sick persons using conventional culture and serological procedures

Bacteriological Examination of Drinking Water and River Water in Community II (Gimo)

The results of the bacteriological examination of drinking water and river water in Gimo will be found in Table 2

The coliform density was found to be lowest in the old supply of drinking water while 800 thermolabile and 300 thermostable coliforms per litre were observed in the new supply of drinking water and 900 thermolabile and 300 thermostable coliforms in a well nearby The Oland river had a coliform density of 20 000 thermolabile and 6 000 thermostable organisms per litre

Direct FA staining of non fluorescent MF gave negative results Two serotypes of FEC 026 B6 and 0114 B? were demonstrated in water from the new drinking water supply after 1 litre of the latter had been filtered through an ordinary white MF and enriched in broth for 14 hours (the two step procedure and ordinary smears) Serotype 0125 B15 were demonstrated in water from a well nearby and from the Oland river A presence of EEC in water from the old supply of drinking water was not demonstrable All these examinations were completed 16–18 hours after the samples had arrived to the laboratory

By means of conventional culture and serology FEC belonging under the serotype 0114 B? was isolated from the new drinking water supply

and serotype 0125 B15 from the well nearby and the Oland river These examinations took 10 days before they were completed

DISCUSSION

Only few papers in the literature have been concerned with the occurrence of EEC in drinking water (2 8 9 12 13) During the summer and autumn of 1965 the drinking water in two communities of Middle Sweden was accidentally contaminated and high densities of coliforms were noted With the use of the MF technique combined with the FA method a rapid demonstration of EEC could be done In one of the communities the contaminated drinking water caused an outbreak of gastroenteritis probably due to EEC This epidemic has been described elsewhere (2)

One of the advantages of the modifications used was the rapidity of the combined MF technique and FA method (4 5) With the technique described in the present paper a preliminary diagnosis could be established within 2-8 hours i e on the same day as the water samples arrived at the laboratory as compared with conventional methods the time thus saved was more than one week

Another advantage of the combined MF technique and FA method was the possibilities thus opened for a quantitation or semi quantitation of the bacterial serotypes demonstrated In an earlier paper (4) it was shown that direct FA staining of non fluorescent MF allowed a quantitation when bacteria were present in concentrations of more than 1 000 organisms per litre of filtered water and with the use of the so called two step procedure a semi quantitation could be made when bacteria were present in concentrations below 1 000 per litre (5) Judging from this EEC 0111 B4 were present in about 1 000 per litre of the drinking water in community I and the other 2 serotypes 026 B6 and 0128 B12 in concentrations of about 100 per litre of water EEC 0111 B4 was the most frequent serotype in faecal specimens from individuals with gastroenteritis during this epidemic outbreak (2) Concerning community II the concentrations of EEC were probably lower than 100 per litre of filtered water

The MF technique combined with the FA method should not of course be used merely for a bacteriological examination of contaminated water It was shown in the present paper however that this technique may be a good complement to conventional bacteriological methods It might for example be of great importance to have a rapid bacteriological information so as to have corrective treatment promptly instituted In this connection it should also be borne in mind that FA techniques used for identifications of EEC may give false positive results owing to serological cross reactions with other bacteria (6) A preliminary diagnosis obtained with FA techniques should therefore

be followed by attempts at confirming the findings with well estab lished conventional methods

SUMMARY

During the summer and autumn of 1965 the drinking water supply in two communities (Community I & II) of Central Sweden was acci dentally contaminated and high densities of coliforms were noted In community I the contaminated drinking water caused an epidemic outbreak of gastroenteritis probably due to enteropathogenic Esche richia coli (EEC) The drinking water in both communities was exam ined with special regard to EEC with the use of the membrane filter (MF) technique combined with the fluorescent antibody (FA) method With these techniques 3 serotypes of EEC 026 B6 0111 B4 and 0128 B12 were demonstrated in community I and the same serotypes were isolated by conventional methods from faecal specimens of affected individuals In community II the serotypes 026 B6 0114 B? and 0125 B15 were demonstrated with the combined MF technique and FA method Two of these serotypes 0114 B? and 0125 B15 were isolated by conventional culture Rapidity was one of the advantages of the combined MF technique and FA method A diagnosis could be arrived at within 1 day while conventional culture and serological procedures took more than 1 week The limitations of the techniques were dis cussed

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A STUDY ON THE WASSERMANN AND TPI ANTIBODIES IN RELATION TO HISTOPATHOLOGICAL FINDINGS IN *T PALLIDUM* INFECTED ANIMALS AND MAN

By

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Received 24 iv 67

The serology of experimental syphilis in various animals particularly the relationship between reagins shown by standard tests for syphilis (STS) and *Treponema pallidum* immobilizing (TPI) antibodies is rather incompletely described in the literature while there is an abundance of information regarding man in this respect For instance it is known that Wassermann reagins appear earlier than immobilizing antibodies in experimental syphilis in human volunteers as well as in accidental human syphilis (*Magnusson et al* 1956 *Hederstedt & Skog* 1964) No corresponding investigations have been performed in experimental animals where susceptibility and pathology are quite different from man

The present work emanated originally from an idea to try to cultivate *T pallidum* under new experimental conditions namely in malignant cells of human origin as well as in normal cells of rabbit origin inoculated into the cheek pouch of the golden hamster *Mesocricetus auratus* Such experiments include steroid treatment of the hamsters to depress the rejection of the implanted tissue which necessitated controls regarding the serological and histopathological effects of inoculated *T pallidum* in nontreated and cortisone treated hamsters These preliminary experiments on the hamster gave somewhat unexpected serological results It was therefore decided to extend the serological as well as the histopathological study to other animal species rabbit and rat In addition the result of a serological study on human syphilis is reported for comparison

MATERIALS AND METHODS

Treponema pallidum The Nichol strain of *T pallidum* was used throughout this investigation The treponemes were extracted from infected rabbit testicles and suspended in TPI test medium (*Hederstedt* 1961) In some inoculation experiments killed treponemes were used The treponemes were killed by exposure to +56 C for 40 minutes followed by the addition of merthiolate (1 1000) and storage over night at +4 C

TPI test and Wassermann reaction (WR) The TPI test and the WR were performed according to methods described earlier (*Hederstedt* 1961) The TPI and the WR titres were expressed as the reciprocal of the highest serum dilution immobilizing at least 50 per cent of the treponemes or causing not more than 50 per cent haemolysis respectively

Human syphilitic cases Sera from 361 human syphilis patients were tested This material represented the total number of untreated serologically reactive cases of early syphilis investigated serologically at the National Bacteriological Laboratory during the period 1959–1963 The criterion for the diagnosis of early syphilis was either the finding of treponemes in the lesion or significantly increasing TPI titre

Hamsters Young adult golden hamsters of both sexes 90–140 g weight were inoculated with 0 1 ml of treponemal suspension *At the beginning of the investigation* the treponemes were injected into the everted cheek pouch under light anaesthesia (0 1 ml per 100 g body weight of a 6 per cent sodium pentobarbital solution administered intraperitoneally) Later using no anaesthetics the suspension was inoculated *under the skin of the neck Prior to inoculation approximately 1 ml of* blood was drawn from each animal by cardiac puncture After varying periods of time the animals were bled to death From each animal 3–5 ml blood was obtained

Rats White male rats weighing 400–500 g were inoculated into one testicle with 0 5 ml of treponemal suspension or intracutaneously into the back about 0 1 ml at 4 6 different sites Blood was drawn immediately prior to inoculation and at varying intervals after the inoculation by cutting the tails of the animals

Rabbits Young male rabbits weighing about 2500 g were inoculated with 1 0 ml treponemal suspension into each testicle Blood was drawn from a marginal ear vein

Pathological examination Most of the animals were dissected Part of the tissues from the site of inoculation as well as of regional lymph nodes were examined under dark field microscope for treponemes and specimens were formol fixed and stained for histological examination

RESULTS

Hamsters Inoculated with Living Treponemes

In one experiment two groups of hamsters eight animals in each group were infected with 9 0 × 10⁶ and 9 0 × 10⁴ treponemes respectively Sera from the 16 animals taken prior to the inoculation were all found to be TPI and WR negative One or two animals from each group were killed at varying intervals (1–12 weeks) after the inoculation and serum was collected All serum samples were examined by the TPI test or the WR in the same days tests

As seen in Table 1 serum from one animal receiving 9 0 × 10⁶ treponemes was found to be reactive five weeks after inoculation with a TPI titre of 5 All animals from both groups exsanguinated six weeks or later after inoculation were found to be TPI positive the highest titre being 160 The WR was not found to be positive in any animal until eight weeks after inoculation Four out of the six animals tested 8–12 weeks after inoculation were found to be WR positive the highest WR titre being 60 There were no obvious difference in serological behavior between the two groups Thus in an inoculum of 9 0 × 10⁴

treponemes there seemed to be an excess of treponemes guaranteein, a high per cent of infectivity of the golden hamster

The TPI was thus found to be positive and the WR negative in sera from nine of the sixteen animals In none of the remaining seven animals the WR was found to be positive and the TPI negative

TABLE 1

TPI and WR Titres in Sera from 16 Golden Hamsters 8 of them Inoculated with 9 0 × 10⁸ (Group I) and 8 with 9 0 × 10⁵ (Group II) Treponemes Each Animal Was Bled to Death and Blood Was Sampled 1–1? Weeks after Inoculation

Time in weeks	WR titre Group I	WR titre Group II	WR titre Group I	WR titre Group II
1	<5	<5	<7 5	<7 5
5	5	<5	<7 5	<7 5
6	5	5	<7 5	<7 5
6	10	10	<7 5	<7 5
7	?0	5	<7 5	<7 5
8	20	20	60	<7 5
9	80	83	<7 5	7 5
12	160	80	15	15

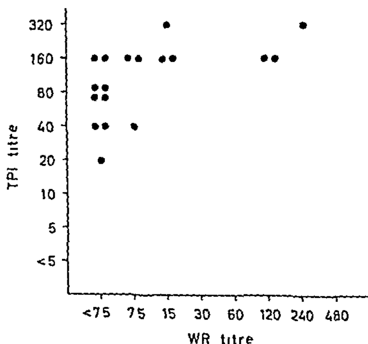


Fig 1

TPI (ordinate) and WR (abscissa) titres in sera from 18 golden hamsters inoculated with 9 0 × 10⁶ ertponemes 12 weeks prior to the sampling of blood.

Another experiment, indicating that the TPI antibodies might develop earlier than the WR reagins in experimental syphilis in the golden hamster is illustrated in Fig 1 Nine out of 18 hamsters all killed 12 weeks after inoculation, were found to be TPI positive but WR negative In two of these animals a TPI titre as high as 160 was recorded

In due time all infected hamsters seem to develop WR reagins as well as TPI antibodies The individual sera from a total of 17 hamsters sampled four to twelve months after inoculation were found to be WR and TPI positive

From the experiments summarized in Table 1 it appears that TPI antibodies can be demonstrated 2–3 weeks earlier than WR reagins in experimental hamster syphilis However the results from the experiment illustrated in Fig 1 suggest that the time from TPI reactivity until WR reactivity varies considerably

TABLE 2

TPI and WR Titres in sera from 13 Cortisone Treated (Group I) and 7 Non Treated (Group II) Golden Hamsters Inoculated with 9.0×10^{8} Treponemes Each Animal Was Bled to Death and Blood Was Sampled 4–12 Weeks after Inoculation

Time in weeks	WR titre Group I	WR titre Group II	TPI titre Group I	TPI titre Group II
4	5	<5	<7.5	<7.5
6	80	40	<7.5	<7.5
8	10	40	<7.5	240
8	40		30	
10	40	320	<7.5	120
10	40		<7.5	
12	20	40	<7.5	120
12	80	40	<7.5	1920
12	160	320	<7.5	240
12	320	640	<7.5	240
12	320		<7.5	
12	320		30	
12	320		60	

Cortisone Treated Hamsters Inoculated with Living Treponemes

Sixteen hamsters pretreated once weekly for three months with 2.5 mg of cortisone (Cortone acetate 25 mg per cc Merck Sharp & Dohme) and eight untreated control hamsters were inoculated with 9.0 × 10 treponemes Three cortisone treated animals died from intercurrent affections The other animals were bled to death at varying intervals (4–12 weeks) after treponemal inoculation and all serum samples were examined by the TPI test and the WR in same days experiments In Table 2 the TPI and WR titres from this experiment are grouped together Four weeks after the inoculation one cortisone treated animal was found to be TPI positive and six weeks or more after inocul

ation all animals—12 cortisone treated and 7 untreated—appeared to be TPI positive Positive WR was demonstrated in all six untreated animals killed eight to twelve weeks after inoculation whereas only three out of eleven cortisone treated were found to be WR positive during the same period

In the present experiment no obvious difference in TPI titres between the cortisone treated and the untreated group was demonstrated whereas the development of WR reagins was depressed or delayed in the cortisone treated group as compared with the untreated group

TABLE 3

TPI and WR Titres in Sera from 14 Hamsters 3 and 14 Days as Well as 4 Months after these Six of these Had Been Inoculated with Living and Eight with Killed Treponemes

	Hamster no	TPI titre 3 days	TPI titre 14 days	TPI titre 4 months	WR titre 3 days	WR titre 14 days	WR titre 4 months
Inoculated with living treponemes	1	<5	5	640	<7 5	<15	480
	2	<10	5	320	<15	<7 5	30
	3	<5	5	†	<7 5	<7 5	†
	4	<5	5	†	<7 5	<15	†
	5	<5	5	†	<7 5	<7 5	†
	6	<5	5	80	<7 5	<7 5	60
Inoculated with killed treponemes	7	10	10	5	<7 5	<7 5	<7 5
	8	<5	†	†	7 5	†	†
	9	5	<5	<5	<7 5	<7 5	<15
	10	<5	10	5	15	<7 5	<7 5
	11	10	10	20	7 5	<7 5	<7 5
	12	<5	5	<5	<7 5	<15	<30
	13	5	5	5	<7 5	<7 5	<7 5
	14	<5	<5	<5	<7 5	<7 5	<7 5

Hamsters Inoculated with Killed Treponemes

Eight hamsters were inoculated with 9 0 × 10^6 killed and six control hamsters with 9 10 × 10^6 living treponemes The treponemes in the two animal groups were derived from the same rabbit Blood was drawn from the hamsters by cardiac puncture 3 and 14 days as well as 4 months after inoculation (after the first puncture one animal inoculated with killed treponemes died and after the second puncture three animals inoculated with living treponemes died) Already three days after inoculation the WR and TPI were demonstrated to be positive in sera from three and four animals respectively inoculated with killed treponemes (Table 3) However the appearance of WR reagins was just transient Fourteen days as well as four months after inoculation no WR reagins were detected in the sera tested while five and four animals respectively had developed TPI antibodies within the same time periods In the control animals WR reagins and TPI antibodies

were not detected until four months after inoculation. A comparison of the results obtained from different experiments indicated that the time interval between the inoculation and the first demonstration of antibodies in the hamster serum seemed to vary with different inocula, e.g. approximately the same number of treponemes derived from different rabbits. A variation in the number of treponemes (9.0×10^{6}–9.0×10^{4}) in inocula derived from one and the same rabbit, however, did not appear to give rise to any variation in the corresponding time interval.

White Rats Inoculated with Living and Killed Treponemes

Eight seronegative white rats were inoculated with 4.5×10 treponemes. Blood was drawn at varying intervals after inoculation and sera were tested with the TPI test and the WR in the same day's experiment (Table 4).

TABLE 4

TPI Titres in Sera 1–90 Days after inoculation into 5 Rats of Living and into 3 Rats of Killed Treponemes

		TPI titres								
	Rat no.	1	2	3	7	14	28	60	90	360 days
Inoculated with living treponemes	1	<5	<5	5	10	20	80	40	10	†
	2	<5	<5	5	10	20	80	40	80	80
	3	<5	5	10	80	40	80	40	20	20
	4	<5	5	10	80	40	80	80	80	40
	5	<5	5	10	80	320	160	320	40	80
Inoculated with killed treponemes	6	<5	<5	5	10	40	80	40	20	5
	7	<5	<5	<5	5	40	40	40	5	5
	8	<5	<5	5	10	80	80	80	20	10

As early as on the second day after inoculation three out of five rats inoculated with living treponemes were found to be positive in the TPI test. On the third day all but one rat inoculated with killed treponemes showed TPI reactivity. After one to two months the TPI titre seemed to reach its maximum and still after one year all sera from the seven animals tested (one animal inoculated with living treponemes died three months after inoculation) were found to be positive in the TPI test, especially, however, in the animals inoculated with killed treponemes in which a regression of the TPI titre was observed. The WR was negative in all serum samples.

In another experiment three rats were inoculated with living, three with killed treponemes. The number of treponemes inoculated into each animal was only 4.5×10^{3}. No TPI reactivity was demonstrated in the blood drawn 4, 7, 14 and 60 days after inoculation.

Rabbits Inoculated with Living Treponemes

Over a period of time 75 TPI negative rabbits received a single injection of 2 ml containing 9 0 $\times$ 10 2 7 $\times$ 10^8 living treponemes per ml The animals were bled to death 2 to 14 days after inoculation WR and TPI tests were performed on sera from blood samples drawn prior to inoculation and at death (Table 5) A pre examination of 100 non treated healthy male rabbits had shown that 82 rabbits were positive in the WR while no TPI reactivity could be demonstrated Only a significant rise of the WR titre (more than one titre step) after inoculation *in comparison with the titre found prior to inoculation was* indicated as 'WR positive' in Table 5 Two to nine days after inoculation a rise in WR titre was demonstrated in 20–30 per cent of the sera tested while there was a rise in 90 and 100 per cent of the sera respectively after 12 and 14 days The TPI test was not found to be positive until eight days after inoculation Twelve and 14 days after inoculation TPI test reactivity was demonstrated in 40 and 80 per cent of the sera respectively

TABLE 5

Number of Rabbits in which a Rise in WR Titre Was Demonstrated or TPI Reaction Had Set in 2–14 Days after Treponemal Infection

Number of days from inoculation to death	Number of rabbits inoculated	WR positive	TPI positive
2	10	2	0
6	10	3	0
7	12	3	0
8	35	10	2
9	8	2	1
12	10	9	4
14	10	10	8

Rabbits Inoculated with Killed Treponemes

In sera from six rabbits inoculated with 9 0 $\times$ 10^7 killed treponemes any TPI reactivity could not be demonstrated in tests carried out 7 14 and 28 days after inoculation Six other rabbits in which WR was *negative prior to the inoculation were inoculated with 3 6 $\times$ 10^8 killed* treponemes Blood was drawn 7 14 and 21 days later Seven days after inoculation the WR was found to be positive in sera from three of the animals Fourteen and 21 days after inoculation only two animals were found to be WR positive In the TPI test all specimens were found to be non reactive On the 21 st day after inoculation each animal was challenged with 9 0 $\times$ 10^7 killed treponemes Seven days later the WR was found to be positive in all six animal the titres ranging between 60–480 The TPI test was found to be positive in sera from four animals the titers being 5–10 Fourteen days after the challenge all ani

TABLE 6

TPI and W R Titres in Sera from Six Rabbits Inoculated with 3.6 × 10 Killed Treponemes 7 14 21 28 and 35 Days Prior to the Sampling of Blood On the 71st Day after the First Inoculation the Animals Were Challenged with 9 0 × 10 Killed Treponemes

Rabbit no	7 days		14 days		21 days		28 days		35 days	
	W R	TPI	W R	TPI	W R	TPI	W R	TPI	W R	TPI
1	<7 5	<5	<7 5	<5	<7 5	<5	60	<5	60	<5
2	30	<5	30	<5	7 5	<5	120	5	60	<5
3	30	<5	30	<5	15	<5	480	<5	240	<5
4	<7 5	<5	<7 5	<5	<7 5	<5	30	5	15	<5
5	15	<5	<7 5	<5	<7 5	<5	240	10	120	5
6	<7 5	<5	<7 5	<5	<7 5	<5	60	5	30	5

mals were still found to be W R positive but only two of them were TPI positive (Table 6)

Serological Findings in Human Early Syphilis

In human early syphilis the W R is most often found to be positive earlier than the TPI test (*Magnuson et al* 1956 *Hederstedt & Skog* 1964) Fig 2 illustrates the W R and TPI titres in 361 cases of human early syphilis It was obvious that cases with high W R titres occurred in connection with low TPI titres

From the present material some conclusions might also be drawn as regards the length of the period of time between the earliest appearance of W R reagins and the appearance of TPI antibodies in early syphilis No attempts to calculate that interval seem to have been reported before Thus in 62 out of the 361 cases the first serum sample was taken at a point when only one of the two reactions was positive In 55 (90 per cent) out of these 62 cases the W R was demonstrated to be positive earlier than the TPI test In 41 out of these 55 cases the TPI test was found to be positive in serum samples collected 2–34 days (average 9 9) later the frequency of the serum sampling was every eighth day or less When the W R was first found to be positive it was impossible in most cases to determine for how many days the patient already had demonstrable WR reagins In that respect the W R titres in the first positive specimen 7 5–960 average 73 can give only limited information When the TPI test was first demonstrated to be positive (2–34 days later) the WR titre was 30–960 average 142 while the TPI titre on that occasion was 5 10 average 5 8 The period required for the WR titre as compared with the TPI titre to reach a maximum in untreated early syphilis usually seems to be shorter but individual variations in this respect may be great (*Hederstedt & Skog* 1964)

In seven cases the TPI test was demonstrated to be positive earlier than the W R Thorough examination of the anamnestic data revealed

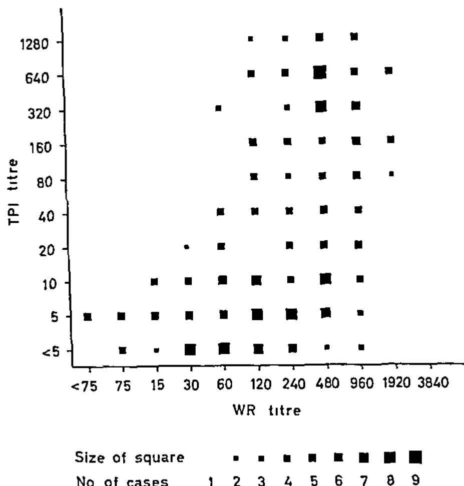


Fig 2

TPI (ordinate) and WR (abscissa) titres in sera from 370 cases of aquired human early syphilis

that three out of the seven patients had a history of medical treatment on account of other apparently infectious diseases during the possible incubation period

Pathological Examinations

In the present investigation any systematic pathological examinations were not performed neither in the cases of human syphilis nor in the rabbits infected with *Treponema pallidum* since the knowledge of the pathology in these two species being well established Thus in both species marked symptoms appears at the site of infection where also mobile treponemes are to be found during a limited period of time

In *the hamsters* the site of infection (the cheek pouch or the skin of the back of the neck) was examined daily for the first four days weekly for up to eight weeks and then every second week up to 14 weeks after inoculation of treponemes At least two animals were examined on each occasion The animals were bled to death and most of the cheek pouch tissue or a piece of the skin (about 1 cm of size) as well as the regional lymph nodes were taken for examination for the presence of treponemes and for histological examination At the site of inoculation mobile treponemes were never seen From the fifth to the twelfth week however mobile treponemes were detected in smears from the lymph nodes Most treponemes observed at the twelfth week and all treponemes observed at the fourteenth week were non mobile Usually only a few treponemes per microscopic field were seen Histological examination of the cheek pouch and skin tissues failed to demonstrate any pathological changes The regional lymph nodes were enlarged up to 2–3 times the size seen in normal untreated animals in the cortisone treated animals no such enlargement was seen Histological examination showed only signs of nonspecific inflammation in the syphilitic animals whether or not they were treated with cortisone

The *white rat* examined the first seven days and 14 30 60 and 90 days after inoculation did not on any occasion show histological alterations or mobile treponemes at the site of inoculation or at regional lymph nodes Immobilized treponemes were detected already one day after inoculation Transfer of popliteal lymph nodes from six rats (three of the rats 10 days after inoculation and three 30 days after inoculation) into rabbit testes did not reveal any dark field positive lesions or TPI antibodies within an observation period of two months

DISCUSSION

The observation that the TPI test in serum from syphilis infected hamsters was found to be reactive earlier than the WR was opposite to the well known serological behavior in man The hamster in contrast to man showed only vague symptoms of disease The question arose if there might exist any correlation between these serological findings and the different pathological symptoms in man and hamster It was suggested therefore that a serological and clinical study of experimental syphilis in other species as well might throw some light on that question In Table 7 the time from the onset of syphilis infection until the earliest demonstration of serum positivity in the WR and the TPI test has been related to the occurrence of syphilitic symptoms in man rabbit hamster and white rat In man and rabbit in whom clinical manifestations of syphilis are clear cut the WR was found to be positive earlier than the TPI test In the hamster in which symptoms are relatively weakly pronounced the WR was found to be positive later than the TPI test and finally in the rat in which no symptoms of

syphilis are perceptible *the WR was never found to be positive* Thus the more pronounced the symptoms of disease the earlier the development of WR reagins as compared with the development of TPI antibodies This observation suggests that the development of WR reagins partly or totally might be a result of the host reaction to the tissue destruction caused by the living treponemes

TABLE 7

The Shortest Period of Time in Days from the Onset of Syphilis Infection until the Demonstration of Serum Reactivity in the WR and the TPI Test in Man (Accidental Syphilis) Rabbit Hamster and White Rat The Occurrence of Syphilitic Symptoms Is Indicated

	WR	TPI	Symptoms of disease
Man	90	30	+
Rabbit	5	7	+
Hamster	56	39	±
Rat	>360	2	—

See text

In order to test this hypothesis the animals were inoculated with killed treponemes Any symptoms of disease were not demonstrable in any of the animals Serologically the white rat reacted mainly as if it had been inoculated with living treponemes *e g* the TPI antibody was detected already within three days WR reagins were not detected even one year after the inoculation These observations as well as the finding of immobilized treponemes at the site of inoculation the day after inoculation of living organisms probably indicate that treponemes are rapidly killed in the white rat possibly within the first 24 hours In the present investigation in contrast to other publications (*Turner & Hollander* 1957) the white rat appeared to be resistant to *T pallidum* This result was also substantiated by the failure to transfer the disease to rabbits by popliteal lymph nodes from syphilis infected rats The fact that unheated serum from normal white rats invariably immobilizes *T pallidum in vitro* within 30–60 minutes (*Hedersledt* 1963) might be of some importance in the resistance of this animal

TPI antibodies in the rat demonstrated as early as 2–3 days after inoculation may have developed as a result of a reaction of the secondary response type which might suggest that the rat normally lodges spirochetes which antigenically are closely related to *T pallidum*

In the hamster the TPI antibody was detected 7–10 days after inoculation of killed treponemes Low titres of WR reagins were detected in some of the animals three days after inoculation but not later on during the observation period of four months When the same number of living treponemes were injected TPI antibodies were not detected until five weeks after inoculation This observation suggests that TPI

antibodies will not develop unless *the treponemes are killed e g have* lost their superficial slimy nonantigenic layer as originally proposed by *Turner & Hollander* (1950) However the fact that the WR was demonstrated earlier than the TPI test upon inoculation of killed treponemes into the hamster while the reverse held true upon inoculation of living treponemes was a most puzzling observation One explanation of this finding might be that heat killing of the treponemes involves that the action of heat may affect the two antigens to varying degree

In contradistinction to the hamster and the rat the rabbit demanded a challenge with killed treponemes to develop demonstrable amounts of TPI antibodies In the rabbit WR *reagins often of high titres* developed in each animal upon inoculation of killed treponemes In agreement with the findings by *McLeod & Magnuson* (1953) the reagins appeared to develop earlier than the TPI antibodies This result does not seem to fit with the assumption that the WR reagins develop as a result of a reaction of the host to its own tissue However in more than 80 per cent of the non infected rabbits natural WR reagins *were demonstrated which might play some role for the divergent serological behaviour of the rabbit*

Magnuson et al (1956) *found that inoculation of killed treponemes* into non syphilitic human subjects had no measurable serological effect In subjects previously treated for syphilis there was however a definite anamnestic response as measured by the TPI test while the WR did not show any significant increase in titre

Cortisone treatment was given to the hamster prior to as well as after inoculation of living treponemes primarily to suppress its homograft reaction The cortisone dose recommended to suppress this reaction was 2–3 mg (*Toolan* 1954) No information seems to be available *as regards the dose of cortisone that will affect the antibody formation* in hamster In the rat (*Berglund* 1956) and in the guinea pig (*Germuth et al* 1952) the lowest cortisone dose required to depress the antibody level was found to be 4 mg per 100 g *body weight However* when such doses were given to hamsters most of the syphilis injected animals died from intercurrent affections In hamsters treated with 2.5 mg cortisone doses (most of the animals survived such treatment) the WR reagins were detected later than *in untreated animals and titres were* lower At the same time the regional lymph nodes in these animals *were less enlarged macroscopically* than in untreated syphilis infected animals However the development of TPI antibodies in the hamster did not appear to be influenced by cortisone treatment A possible explanation of these results is that the dose of cortisone used was to small to depress the development of antibodies but great enough to depress the tissue reaction Thus *the formation of TPI antibodies would* be unaffected while the formation of the WR reagins if developed as a result of host tissue *reaction would be suppressed*

A minor study by *Turner & Hollander* (1954) on the effect of cortisone on syphilis serology in the rabbit indicated that the W R titre was slightly lowered in animals receiving larger doses of cortisone while no corresponding reduction in treponemal immobilizing antibody was noticed

A detailed study of the cortisone effect on the W R reagins (natural occurrence included) and the TPI antibodies in various species would be a desirable testing of the above explanation of the present serological results obtained in experiments on cortisone treated hamsters

The nature of the Wassermann reagins is still a matter of discussion Thus according to some frequently quoted theories the W R reagins have been considered to be antibodies against lipoids of the treponeme (*Eagle* 1937) or auto antibodies (*Weil & Braun* 1909) or antibodies against tissue haptens combined with treponemal proteins (*Sachs Klopstock & Weil* 1925) The observation by *Schipper & Chesney* (1950) that the titre of W R reagins was directly correlated to the number and extent of syphilitic lesions suggested that WR reagins developed as the result of a host tissue reaction to the parasite This observation is in agreement with the results obtained in the present investigation viz that the W R reagins were demonstrated earlier than the TPI antibodies in species developing marked histopathological lesions while the reverse held true in species with no or vague symptoms

It has often been suggested that the lipid antigen taking part in the WR is ubiquitously distributed in nature In the present investigation treponemal lipids as well as lipids from unavoidable rabbit testicle tissues might also have given rise to the development of W R reagins

Extrapolation of results obtained in animals to man is often a doubtful task However the results obtained in the present investigation especially in the cortisone experiments suggest that in man at least such WR reagins as develop early during the disease are mainly the result of a host reaction to tissue destruction caused by the treponemes

In the animal experiments the TPI antibodies regularly developed after inoculation of killed treponemes It seems plausible that these antibodies are formed upon treponemal antigen stimulation

As long as cultivation of *T pallidum in vitro* is unsuccessmul *e g* as long as a pure suspension of *T pallidum* free from rabbit tissue debris is not available it will certainly be difficult to get an unambiguous answer as regards the nature of the W R reagins

SUMMARY

In hamster rabbit and white rat the period of time required for the development of Wassermann reagins and TPI antibodies upon inoculation of living or killed *T pallidum* was determined and coexisting histopathological symptoms were examined The results were compared with corresponding findings observed in cases of acquired syphilis in

man The Wassermann reagins were demonstrated earlier than the TPI antibodies in human syphilis and in rabbit inoculated with living *T pallidum* both species showing clear cut symptoms of the disease In the hamster in which only small histopathological alterations were noted TPI antibodies were detected earlier than the reagins Upon treatment with relatively small cortisone doses the development of WR reagins in the hamster was suppressed while the production of TPI antibodies was unaffected Although TPI antibodies were found in the white rat symptoms of disease and reagins did not appear Upon inoculation of killed treponemes all animals produced TPI antibodies The rabbit and some of the hamsters developed reagins In 82 per cent of the non infected rabbits naturally occurring Wassermann reagins could be demonstrated

The results obtained in the present investigation suggest that Wassermann reagins to appear early in human syphilis develop mainly as a result of a host reaction to tissue destruction caused by the treponemes while the production of TPI antibodies is caused by treponemal antigen stimulation

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EFFECT OF TRYPSIN ON COMPLEMENT COMPONENTS IN NORMAL HUMAN SERUM

By

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In the present work it was found that incubation of normal human serum with trypsin in an amount corresponding to less than 10–20 per cent of the normal trypsin inhibitor capacity of serum activated C 1 to C 1 esterase and reduced the concentrations of C 1 esterase inhibitor C 4 and C 2 Under these conditions β_{1C} was converted and thereby similar observations by *Laurell, C B* and *Ganrot, P O* (personal communication) were confirmed

MATERIAL AND METHODS

Normal human sera Blood was obtained from normal subjects The serum was left for 2 hours at room temperature and separated It was then stored at —60 C.

N acetyl L-tyrosine ethyl ester (ATEe) from Calbiochem Luzern Switzerland

Agarose from L Industrie Biologique Francaise S A Gennevilliers France

Sodium ethylendiamine hydroclonde (Na H EDTA) was added to the serum to final concentrations of 0 001 M and 0 01 M respectively

Trypsin (Novo A S Copenhagen Denmark) Chrystalized

Trypsin treatment of normal sera Ten mg of trypsin was dissolved in 1 ml of 0 0025 N hydrochloric acid Fresh mixtures were prepared for each experiment To one ml of serum 0 025 ml of the trypsin solution (0 25 mg) was added and the mixture was incubated at 37 C for 45 minutes A control tube with serum containing a corresponding volume of hydrochloric acid was incubated for the same period of time at 37 C

Total complement (C) and complement component titrations were performed according to *Pillemer et al* (1956) The smallest amount of serum producing 50 per cent haemolysis was taken as one unit

C 4 inactivating capacity was determined as described by *Laurell & Siboo* (1966)

Acetyl tyrosine ethylester (ATEe) hydrolyzing capacity was determined with the pH stat (Radiometer Copenhagen Denmark) as described by *Laurell et al* (1965) with the exception that the buffer used was 0 005 M Enzyme activity was expressed in units according to *Levy & Lepow* (1959)

C 1 esterase inhibitor was measured according to *Laurell et al* (1965) The unit of C1 esterase inhibitor is defined according to *Levy & Lepow* (1959)

Conversion of β_1C globulin in serum was determined by antigen antibody cross d electrophoresis (*Laurell* 1965) No Ca++ was added to the buffers used The anti um used reacted with β_1C globulin and its conversion products and also contained anti bodies to the β lipoproteins The degree of conversion was estimated by plan m t The amounts of β_1C and of β_1C conversion products was expressed in p t t th total surface of converted and non-converted anti en in each exp ime t No or

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rection was made for the difference in diffusion rate between β_1C and the conversion product

Gel filtration on Sephadex G 200 was performed according to *Laurell & Siboo* (1966)

RESULTS

Effect of Trypsin on Total Complement and Complement Components

Individual normal human sera were incubated with 0 025 ml of the trypsin solution per ml of serum (0 25 mg/ml serum) A control with the corresponding volume of 0 0025 N hydrochloric acid was prepared The total complement and complement components were measured In repeated tests a slight decrease of the total complement was found in serum treated with trypsin C 1 activity was at most slightly reduced C 4 activity was reduced to about 25 per cent and C 2 activity to about 50 per cent When serum was heated at 52 C for 30 minutes before treatment with trypsin no reduction in C 4 activity was demonstrated An illustrative example of these experiments is given in Table 1

TABLE 1
Effect of Trypsin on C'1 C'2 and C'4 of Normal Human Serum

	Units per ml		
	C'1	C 2	C'4
Serum treated with trypsin	1200	150	150
Serum control	1200	300	600
Serum heated 52 C 30 min then treated with trypsin	10	10	300
Serum heated 52 C, 30 min	10	10	300

Gel Filtration on Sephadex G 200 of Normal Serum Treated with Trypsin

Normal serum treated with trypsin 0 25 mg/ml serum at 37 C for 45 minutes was dialysed against TRIS buffered saline (TBS) with an ionic strength of 0 15 pH 7 4 and containing 0 001 M EDTA Gel filtration was performed with TBS EDTA as eluent The fractions were tested for C 4 inactivating capacity as an expression of C 1 esterase The fractions located between the second and third peaks had the power to inactivate C 4 (Fig 1) After tenfold concentration of these fractions ATEe hydrolysing activity was also demonstrable

Serum not treated with trypsin was investigated in the same way In accordance with earlier findings (*Laurell & Siboo* 1966) the fractions obtained did not inactivate C 4 or hydrolyse ATEe

When gel filtration was performed on untreated or trypsinised serum in the presence of Ca the first peak showed destruction of C 4 (see *Laurell & Siboo* 1966) indicating activation of C 1 to C 1 esterase in macromolecular form No inactivation of C 4 was found with the fractions from the second or third peak

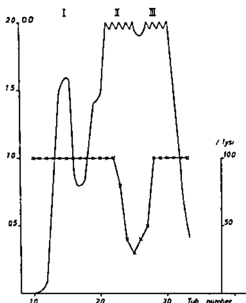


Fig 1

Gelfiltration on Sephadex G 200 of trypsin treated normal human serum The serum was dialysed against triethanolamine buffer with an ionic strength of 0 15 pH 7 4 containing 0 001 M EDTA The same EDTA containing buffer was used as eluent ●——●——● O D 280 mμ ×——×——× C 4 destroying activity

Effect of Trypsin on C 1 Esterase Inhibitor of Normal Serum

After similar incubation of normal serum with trypsin the C 1 esterase inhibitor was reduced to 20–35 per cent of the original con centration (Table 2) Trypsin also reduced the C 1 esterase inhibitor when incubated with serum containing EDTA in a final concentration of 0 001 M or 0 01 M

TABLE 2

Effect of Trypsin on C'1 Esterase Inhibitor in Normal Human Sera

	C 1 esterase inhibitor U/ml						
	Serum VII	Serum VIII	Serum IX	Serum XI	Serum XII	Serum XIII	Serum XV
Serum not treated with trypsin	11	18	19	16	11	16	14
Serum treated with trypsin	4	6	5	3	2	5	4
EDTA serum (final concentration 10^{-3} M)							
not treated with trypsin	12	17	19	13	1	16	13
treated with trypsin	2	3	5	2	1		3
EDTA serum (final concentration 10^{-2} M)							
not treated with trypsin	17	21	23				
treated with trypsin	4	8	3				

Effect of Trypsin on β_1c Globulin in Serum

Incubation of normal sera with trypsin resulted in the conversion of β_1c. In most sera a spontaneous β_1c conversion was observed which was not appreciably increased when the sera were preheated at 37° C for 30 minutes (Fig 2 and Table 3). The reproducibility of the results obtained with this method is seen in Table 4.

TABLE 3

β_1c Conversion on Trypsin Treatment of Normal Human Sera

	β_1c conversion in per cent			
	Serum incubated with		Serum prewarmed 37° C 30 min and then incubated with	
	trypsin	HCl	trypsin	HCl
Serum I	35	6		
Serum II	44	0		
Serum III	46	5	34	6
Serum IV	67	15		
Serum V	48	6		
Serum VI	47	15	45	21
Serum VII	47	10	39	20
Serum VIII	81	17	89	18

In sera heated at 52° C for 30 minutes and then incubated with trypsin a small increase of β_1c conversion was demonstrated compared to the spontaneous conversion in the control sample. When the sera were heated at 56° C for 30 minutes addition of trypsin did not increase the conversion.

On incubation with trypsin of normal sera containing EDTA in a

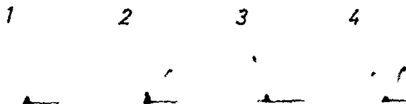


Fig 2

Crossed antigen antibody electrophoresis of normal human serum

1 Normal serum I incubated with HCl
2 Normal serum I incubated with trypsin
3 Normal serum I (heated 37° 30 min) then incubated with HCl
4 Normal serum I (heated 37° 30 min) then incubated with trypsin

Precipitation peak to the left = β_1c
Precipitation peak to the right = converted β_1c
Intermediate precipitation peak = β lipoprotein

final concentration of 0 001 M the conversion of β_{1C} was the same as that obtained with normal serum treated with trypsin in the absence of EDTA (Table 5 and Fig 3) This dose of EDTA completely inhibited immune haemolysis of sensitized sheep red cells with the sera tested *On the other hand trypsin treatment in the presence of* 0 01 M EDTA reduced the conversion of β_{C_1} But this inhibition of β_{1C} conversion was never complete The spontaneous conversion was inhibited or markedly reduced in the presence of 0 01 M EDTA

TABLE 4

Reproducibility of Determination of β_1C Conversion by Crossed Antigen Antibody Electrophoresis

	β_1C conversion in per cent in serum incubated with	
	Trypsin	HCl
Normal serum X		
first test	48	6
second test	47	13
Normal serum XI		
first test	50	15
second test	47	17
Normal serum VIII		
first test	81	17
second test	85	15
Normal serum XI		
first test	45	28
second test	50	18

TABLE 5

β_1C Conversion on Trypsin Treatment of Normal Sera in Absence and in Presence of EDTA

	β_1C conversion in per cent Normal serum		
	X	XI	V
Serum + tryp in	48	47	72
Serum + HCl	6	17	9
Serum + EDTA (final concentr 0 001 M) + trypsin	50	50	60
Serum + EDTA (final concentr 0 001 M) + HCl	10	27	43
Serum + EDTA final concentr 0 01 M) + trypsin	28	31	40
Serum + EDTA (final concentr 0 01 M) + HCl	0	8	0

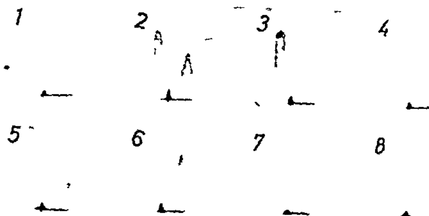


Fig 3
Crossed antigen antibody electrophoresis of normal human serum
1 Normal serum + HCl
2 Normal serum + trypsin
3 Normal serum + EDTA 10 M (fin conc) + HCl
4 Normal serum + EDTA 10 M (fin conc) + trypsin
5 Normal serum + EDTA 10 ³ M (fin conc) + HCl
6 Normal serum + EDTA 10 ³ M (fin conc) + trypsin
7 Normal serum + NaCl + HCl
8 Normal serum + NaCl + trypsin
Precipitation peak to the left = β_1C
Precipitation peak to the right = converted β_1C
Intermediate precipitation peak = β lipoprotein

DISCUSSION

Addition of a small amount of trypsin to normal human serum resulted in inactivation of C 4 and C 2 and in conversion of β_{1C} (C 3) to an electrophoretically faster product Inactivation of C 4 was prevented when the serum was heated at 52 C for 30 minutes before the addition of trypsin When the serum was heated at 56 C for 30 minutes addition of trypsin did not result in conversion of β_{1C}

Since C 1 esterase is known to destroy C 4 and C 2 haemolytic activity (*Lepow et al* 1956 *Muller Eberhard & Lepow* 1965) and to generate the β_{1C} converting activity of C 4 and C 2 in the fluid phase (*Pondman* 1964 *Da Silva & Lepow* 1966 *Muller Eberhard et al* 1967) the question arises whether the effect of trypsin on C 2 C 3 and C 4 could be explained by activation of C 1 to C 1 esterase This possibility is supported by recently published work of *Ratnoff & Naff* (1967) who demonstrated that purified C 1s can be converted to C 1 esterase by trypsin

In the present investigation the amount of trypsin added to whole serum was only 10–20 per cent of the amount normally inhibited by serum (for references see *Eriksson* 1965) Nevertheless this amount was sufficient to inactivate C 2 and C 4 and to convert β_{1C} to an elec

trophoretically faster component The fact that this effect of trypsin disappeared when the serum was heated at 56 C for 30 minutes indicated that trypsin did not act on the components directly but rather exerted its effect by a serum factor sensitive to heating It is suggested that this heat labile serum factor is identical with C 1 which can be activated in serum by even small amounts of trypsin This hypothesis may be supported by the finding that on incubation of serum with trypsin the C 1 esterase inhibitor concentration decreased to 20–35 per cent of its initial value This decrease might be due to a consumption of the inhibitor by C 1 esterase But the possibility remained that the C 1 esterase inhibitor was hydrolysed by trypsin However such an effect of trypsin would not explain the activation of C 1 esterase *Lepow et al* (1965) showed that C 1 esterase inhibitor delayed but did not prevent the activation of C 1 to C 1 esterase

Serum treated with trypsin and subsequently separated by gel filtration on Sephadex G 200 in the presence of EDTA exhibited C 4 destroying activity in an area located between the second and third protein peak eluted No such activity could be demonstrated in serum separated in the presence of EDTA without preceding exposure to trypsin This finding provides evidence for the generation of C 1 esterase activity on trypsin treatment of serum

In view of the indications that β_{1C} conversion is a result of trypsin activation of C 1 esterase and subsequent activation of C 4 and C 2 in the fluid phase it was surprising to find that conversion of β_{1C} was abolished only to about 50 per cent in the presence of 0 01 M EDTA In the presence of 0 001 M EDTA the conversion of β_{1C} was the same as in the absence of EDTA

This leads to the question whether the conversion of β_{1C} by small amounts of trypsin may be brought about also by mediating factors others than those represented by C 1 C'4 and C 2 Such a system was recently demonstrated by *Muller Eberhard et al* (1966) who showed that a factor of cobra venom interacted with a serum factor forming a β_{1C} converting principle This converting system is however inhibited by 0 01 M EDTA

A possible cause of the β_{1C} conversion besides the early factors of the complement system and the cobra factor system might be the kallikrein system Kallikrein was found to be activated by trypsin possibly owing to the destruction of an inactivator as suggested by *Werle* (1955) Since the C 1 esterase inhibitor also inhibits kallikrein (*Kagan & Becker* 1963) the possible role of the consumption by C 1 esterase of this inhibitor in the activation of the kallikrein system should be studied further So far no investigation has been made of β_{1C} as a possible substrate of kallikrein

SUMMARY

1 Addition to normal human serum of trypsin in an amount of less than 10-20 per cent of the trypsin inhibiting capacity resulted in activation of C 1 to C'1 esterase partial inactivation of C 4 and C 2 and partial conversion of β_{1C} C 1 esterase inhibitor decreased to 20-35 per cent the normal value

2 *Addition of 0 001 M EDTA to normal serum before trypsin treatment did not inhibit conversion of* β_{1C}

3 Trypsin treatment of *normal serum in the presence of 0 01 M* EDTA reduced the conversion of β_{1C} but never inhibited it completely

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A NEW EPIDEMIC PHAGE TYPE OF *STAPHYLOCOCCUS AUREUS*

1 *The Experimental Typing Phage 6557*

By

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During the years 1962–65 the Danish Staphylococcal Registration at Statens Seruminstitut recorded outbreaks in several hospitals of infections due to non typable strains with certain properties in common The strains were distinguished by their multiple antibiotic resistance and particularly by their resistance to erythromycin neomycin and bacitracin Most of them were Tween(80) negative i e they failed to give a lipase reaction on Tween 80/calcium agar (*Sierra* 1957) They were non typable with the international basic set of typing phages (*Blair & Williams* 1961) but phages able to lyse staphylococci of group III frequently caused inhibition reactions In our search for a new typing phage in this situation we concentrated on one designated as phage 6557 the properties and application of which will be reported presently

Preliminary investigations (*Bülow & Rosendal* 1964) showed that out of a total of 560 strains non typable with the conventional typing phages 382 could be lysed by phage 6557 Only 2 per cent of these strains were either sensitive to all antibiotics (sens) or resistant to penicillin (P) or to both penicillin and streptomycin (PS) whereas about 31 per cent were resistant to penicillin streptomycin and tetracycline (PST) including some strains also resistant to chloramphenicol (PSTC) and about 64 per cent were resistant not only to penicillin streptomycin and tetracycline but also to erythromycin (PSTE) including 10 per cent which were also resistant to chloramphenicol (PSTCE)

Among the 178 strains not lysed by phage 6557 66 per cent were either sens (35 per cent) or P or PS about [illegible]0 per cent PST or PSTC, and 5 per cent only PSTE or PSTCE Resistance to neomycin and bacitracin was not investigated

Thus these preliminary investigations suggested that phage 6557 used as a typing phage might be able to divide the non typable strains into two groups with different antibiotic resistance and possibly be useful in the new staphylococcal situation

In other countries the same problems had arisen outbreaks of staphylococcal infections due to non typable strains and atypical group III strains were reported from U S A (*Thomas et al* 1960 *Allemeier et al* 1963 *Lavine et al* 1963) from Great Britain (*Temple & Blackburn* 1963 *Jacobs et al* 1963 *Turner* 1963 *Jevons & Parker* 1964) and from Canada (*Comtois & Bynoe* 1963 *Comtois* 1965) Most of the

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strains were reported to be highly resistant to several antibiotics including neomycin In many laboratories efforts have been made to isolate suitable phages for the typing of such strains The experimental phages investigated comprise phage D (*Wallmark & Finland* 1961) phage 77Ad (*Jevons & Parker* 1964) phages B5 and 12100 (*Jevons & Parker* 1964) isolated by *Porter* and by *Clarke* respectively phage UC18 (*Thomas et al* 1960) isolated by *Hill* (University of Cincinnati Ohio) phage SF isolated by *Hurst* (1965) and phage 116 isolated by *Needham* at the Mayo Clinic Rochester Minn (*Comtois* 1965) The first five phages were used by *Jevons & Parker* (1964) in a survey of non typable strains from different geographical areas (U S A Norway Switzerland and Peru) In spite of a certain similarity (inhibition reactions with the phages lysing strains of group III) the strains showed an unexpected heterogeneity as regards susceptibility to lysis by the five phages none of which were found entirely satisfactory as typing phages

In a study by *Hurst* (1965) 55 out of 80 non typable hospital strains were lysed by phages B5 77Ad UC18 SF and in some cases also by phage D According to *Hurst* this pattern may define a previously unrecognized epidemic strain in North America This suggestion is supported by *Lactae et al* (1965) who described a hospital epidemic due to multiple resistant staphylococci not typable by means of the international basic set of typing phages but able to be lysed by the phages D UC18 and SF

The first four phages mentioned and phage 116 were applied to collections of epidemiologically related non typable hospital strains from different parts of Canada by *Comtois* (1965) Most of the strains could be identified by means of the phages B5 and 77Ad furthermore some of the strains lysed by these two phages could be differentiated using the phages D UC18 and 116

In Denmark phage 6557 has been used routinely as a typing phage for epidemiological purposes since April 1963 in the following studies the results obtained will be reported The present paper will give an account of phage 6557 and three international experimental phages (D B5 and 77Ad) Furthermore a comparison of the lytic abilities of these phages has been made partly by using selected test strains partly by applying the phages to a number of epidemiologically unrelated staphylococcal strains with a common characteristic property i e they are not lysed by the typing phages from the basic set at RTD However these strains could be divided into several sub groups according to antibiotic resistance and susceptibility to some phages of the basic set at 1000 × RTD and to some pool phages at RTD The investigation was intended to provide a basis for the choice of a phage or phages suitable in the given situation

MATERIAL AND METHODS

The material referred to as the total material has been described by *Rosendal et al* (1963)

Since 1960 phage typed *Staphylococcus aureus* strains have been registered at Statens Seruminstitut which serves as a typing centre for the whole of Denmark The registration comprises the first strain isolated from patients and staff members of hospitals and the number registered per year amounts to 12 000–14 000 (*Rosendal & Bülow* 1967)

The strains have been phage typed (*Rosendal et al* 1963) and examined for Tween 80 splitting enzyme by the method described by *Jessen et al* (1963) using Sierra s medium (1957) Since 1962 all strains have been examined for resistance to mercuric chloride (*Moore* 1960)

Antibiotic sensitivity tests have been performed in several laboratories (*Rosendal et al* 1963) Furthermore since November 1961 the "prediffusion method" as described by *Thomsen* (1962 1964) has been used routinely at Statens Seruminstitut The diameter of the inhibition zone has formed the basis for the division between sensitive and resistant strains

1079 strains included in the total material from 1963 and 1964 and non typable (NT) with the standard set of typing phages have been used for evaluating the suitability of the following experimental phages

Phage 6557 isolated by the chloroform method (*Kjems* 1955) from a *Staphylococcus aureus* strain (specimen No 6194/1960 C1) and propagated (soft agar l r

method *Swanstrom & Adams* 1951) on strain (specimen No 6557/1960–61) For further details see Table 1

The phages D 77Ad and B5 the origins of which were described in the introduction to this paper were received from The Cross Infection Reference Laboratory Colindale London The two last mentioned phages have later (Moscow Congress 1966) been designated 84 and 85 respectively and included in the basic set of typing phages

RESULTS

1 *The New Experimental Phage 6557*

Donor strain and propagating strain Phage 6557 is a temperate phag found in a culture of *Staphylococcus aureus* isolated from a pleural empyema (bacteriological specimen No 6794/1960–61) The donor strain has the phage type 83A as determined by the standard procedure it is furthermore lysed by pool phage 31B at RTD and at 1000 × RTD by the basic set phages 29 47 54 75 77 and by pool phage 52B

A strain (PS 6557) with very similar properties also isolated from a specimen of pleural empyema (bacteriological specimen No 6557/1960–61) was chosen as the propagating strain Typed by the basic set of phages it has the type 83A it is lysed at RTD by pool phage 31B and by the experimental phages D 77Ad and B5 and it shows inhibition phenomenon with the phages 7 47 54 and 77

TABLE 1

Properties of Donor Strain and Propagating Strain for Phage 6557

	Donor strain	Propagating strain (PS 6557)
Phage type at RTD	83A/31B	83A/6557/31B
Phage type at 1000 × RTD	83A/29/47/54/75/77/31B/52B	83A/6557/77Ad/B5/31B
Resistance to mercuric chloride (Hg)	+	+
Tween 80 reaction (TW)	—	—
Sensitivity to		
penicillin (P)	resistant	resistant
streptomycin (S)	resistant	resistant
tetracyclines (T)	resistant	resistant
erythromycin (E)	sensitive	sensitive
neomycin (N)	sensitive	sensitive
bacitracin (B)	sensitive	sensitive

The donor strain and the propagating strain are resistant to penicillin streptomycin and tetracycline but sensitive to erythromycin chloramphenicol neomycin and bacitracin The strains are resistant to mercuric chloride (Hg+)and do not form extracellular Tween 80 splitting enzyme i e they are Tween negative (TW —)

The properties of the two strains are summarized in Table 1

TABLE 2

Lytic Spectrum of Four Experimental Phages (Host Range)

Phages	3A	3B	3C	6	7	29	29A	31/44	42B/47C	42C	42E	44A	47	47B	52	52A/79	53	54	71	75	77	80	2009	8719	Serological group
D	1	1		5	5			3	1			2	5	1				4		5					F
77Ad	1	1		5	5		3	3	2				5		1		5	5		5	4				F
B5	1			5	5		2	2			3		5		1	3	5	5		5	5				B
6557				5	5		3	2			2	2	5				5	5		3	5				B

Strength of phage reaction
5 = maximum titre on homologous propagating strain
4 = 10^{-1}–10 of titre on the propagating strain
3 = 10^{-3}–10^{-4} of titre on the propagating strain
2 = 10^{-5}–10^{-6} of titre on the propagating strain
1 = very weak lysis

Serological grouping Phage 6557 was found to belong in the serological group B According to the results given by the The Central Public Health Laboratory Colindale London (*Parker* 1964) the phages D 77Ad and B5 belong in the serological groups F, F and B respectively

Lytic spectrum The results of a determination of the lytic spectrum of phage 6557 are listed in Table 2 where it is compared with the spectra of phages D 77Ad and B5 as determined by The Public Health Laboratory Colindale (*Parker* 1964)

It is seen that only minor differences in host range are found between the phages 77Ad B5 and 6557 whereas phage D differs from the other three phages in that it is unable to lyse PS 29A PS 53 and PS 77

2 *The Suitability of Phage 6557 as a Typing Phage*

Phage 6557 and phages D, 77Ad and B5 applied to a material of NT strains In order to compare their suitability for typing purposes these four phages mentioned above were applied to a material of 1029 strains non typable by the standard procedure with the basic set of phages at RTD The strains were a consecutive series of NT strains received during the period from the routine typing laboratory

The antibiotic sensitivity of each strain was recorded when available and the lytic reactions were tested with the following phages the four experimental phages at RTD the pool phages at RTD and at 1000 × RTD and the basic set of phages at 1000 × RTD The typing procedure divided the material into the following categories

A	Lysed by one or more of the experimental phages only	350 strains
B	Lysed by one or more of the experimental phages *and*	
	1) typing phage 83A only (1000 × RTD)	115 strains
	2) typing phage 53 and/or 77 only (1000 × RTD)	95 strains
	3) several phages of group III (1000 × RTD)	43 strains
	4) phages of group I and group III (1000 × RTD)	70 strains
	5) pool phages 31B and/or 52B (RTD)	99 strains
	6) pool phages 42B/47C (1000 × RTD)	7 strains
C	Not lysed by any of the phages	250 strains
	Total	1029 strains

In Table 3 the above mentioned groups have been subdivided according to antibiotic patterns

A) Of the 350 strains lysed by one or more of the experimental phages only 78 per cent are lysed by all four experimental phages Furthermore each of the phages lyses the following percentages of the strains D 7 per cent 77Ad 17 per cent B5 15 per cent and 6557 about 6 per cent

B 1) The 88 strains with known antibiotic sensitivity lysed by phage 83A at 1000 × RTD only distinguish themselves as a unit by including many strains (88 per cent) with the combined antibiotic pattern PST In all respects the strains resemble type 83A strains lysed at RTD (*Rosendal & Jessen* 1964) also as regards erythromycin resistance (9 per cent) Practically all the strains in this category are lysed by all four experimental phages

B 2) The majority (95) of the 138 strains lysed at 1000 × RTD by group III phages are lysed only by the typing phages 53 or 77 or both Of the 91 strains with known antibiotic sensitivity 66 per cent have the combined antibiotic pattern PST(C)E 26 per cent have the pattern PST(C) and as few as 8 per cent are of lower resistance or fully sensitive

Most of the strain (88 per cent) are lysed by all four experimental phages Furthermore each of the phages lyses the following percentages Phages D 77Ad and B5 6 per cent phage 6557 5 per cent

B 3) The remaining 33 strains with known antibiotic sensitivity lysed at 1000 × RTD by several group III phages are on the whole resistant to fewer antibiotics than the strains lysed by the phages 53 and/or 7 only Fourteen strains (42 per cent) are either fully sensitive or resistant to penicillin alone or in combination with streptomycin or tetracycline 12 strains (36 per cent) are PST and 7 strains (21 per cent) are PST(C)E

Only 33 per cent of the 43 strains are lysed by all the experimental phages each of the phages furthermore lyses the following percentages D 40 per cent 77Ad 25 per cent B5 15 per cent and 6557 33 per cent

It appears that strains lysed by group III phages at 1000 × RTD do not form a homogeneous entity but form at least two categories B 2 and B 3) Nevertheless if the 124 strains with known antibiotic

TABI

Distribution of 772 Non typable (NT) and Various Group III Strai

Antibiotic pattern	Sensitive P PS or PT						PST (+C = 13 %)					
Category	A	B (1)	B (2)	B (3)	B (4)	B (5)	A	B (1)	B (2)	B (3)	B (4)	B (5)
Phage type or group	NT	83A	53/77	III	I + III	31B/52B	NT	83A	53/77	III	I + III	31B/52B
D	4		3	5	6	31				1		2
77Ad				3	4		6		2	2	7	5
B5	1		2	2			1	--		1		3
6557	1			1	1							
D/77Ad	1						1				--	--
D/6557			1	3		44			1			5
77Ad/B5		--					8					
B5/6557	1			1	5			1			2	
77Ad/6557					3					--	1	
D/77Ad/B5	1						8	1				
D/B5/6557				1	5	1	1				1	
D/77Ad/6557								1		2		1
77Ad/B5/6557	1				1		1			2		
D/77Ad/B5/6557	9	3	1	--		1	91	74	21	4	9	
Total	19	3	7	14	27	77	117	77	24	12	20	16
Percentage	6	3	8	4?	43	8?	40	87	26	36	32	17

Percentage of strains with known antibiotic resistance within the same phage group
Categories A B(1) B(2) B(3) and B(4) tested with concentrated phage suspensions 1000 × RTD Category B(5) tested at RTD

resistance are considered together they are characterized by including many strains (54 per cent) with the combined antibiotic resistance PST(C)F This is in contrast to the ordinary group III strains lysed at RTD of which only 2 5 per cent are PST(C)F

For the combined categories B 2) + B 3) the percentage of strains lysed by all four experimental phages is 68 per cent in addition 17 per cent are lysed by phage D 12 per cent by phage 77Ad 9 per cent by phage B5 and about 14 per cent by phage 6557

B 4) This category comprises 70 strains lysed by typing phages of group I as well as group III at 1000 × RTD The antibiotic patterns are PST(C)F 25 per cent PST(C) 32 per cent lower resistant or fully sensitive 43 per cent

Only 30 per cent of these strains are lysed by all the experimental phages in addition 24 per cent are lysed by phage D 27 per cent by phage 77Ad 31 per cent by phage B5 and 38 per cent by phage 6557

It is seen that the strains in this category are very similar to those in the preceding category (B3) as regards antibiotic resistance and susceptibility to the experimental phages

B 5) The characterization "non typable of the 99 strains lysed by

cording to Their Susceptibility to Four Experimental Phages

PSTE (+C = 19 %)						Antibiotic sensitivity unknown						
A	B (1)	B (2)	B (3)	B (4)	B (5)	A	B (1)	B (2)	B (3)	B (4)	B (5)	
NT	83A	53/77	III	I + III	31B/52B	NT	83A	53/77	III	I + III	31B/52B	Total
									2	2		56
						6		1				36
4		1		1							1	17
									1	1		5
	-					2						4
1		1		1		-			2		5	64
2		1				8		-				19
							1			1		12
				1							-	5
2		-				2						14
				1	-	1				1		12
-			1	-								5
5		2	1	1	-	7				1		22
143	8	55	5	11		31	26	3	5	1		501
157	8	60	7	16	0	57	27	4	10	7	6	772
54	9	66	21	25	0							

the group III pool phages 31B and/or 52B at RTD is evidently a mere logical sequence of the conventional inclusion of these phages in the pools and not in the basic set the strains are typable by means of diluted phage suspensions Actually they constitute an entity distinct from the remainder of the non typable strain material as regards anti biotic resistance Only 16 (17 per cent) of these strains have the com bined antibiotic pattern PST 83 per cent have a lower antibiotic re sistance—and these mostly resistant to P only (*i e* about 70 per cent) whereas the remainder are either sensitive or PS or PT The very low incidence of strains with the combined antibiotic pattern PST is even more striking in view of the fact that 8 of the 16 strains seem to form a separate unit being lysed by one of the phages 77Ad or B5 Only one of all the 99 strains was lysed by all four phages

B 6) A small but remarkable collection of 7 strains lysed by the pool phages 42B and 47C at 1000 × RTD may be considered as a distinct entity There is probably no epidemiological connection between these strains Five of the strains are known to be resistant to penicillin streptomycin and tetracycline The 7 strains were only lysed by the experimental phage 77AD at RTD

C) This category of 250 strains not lysed by any of the available phages corresponds to the 278 strains mentioned previously (*Bulow & Rosendal* 1964) and *in the introduction of this paper* they will not be commented on further

An evaluation of phage 6557 Summing up we may say that the 1029 non typable strains do not form a *homogeneous* collection of strains but fall into several more or less distinct categories the last three of which must be excluded if we are to have a material of strains we can use to assess the value of 6557

Category B 6) is excluded because the strains are not really non typable with the conventional typing phages but form a special unit within the group III staphylococci (type 31B/52B)

Categories B 6) B 7) and C) have been omitted from the following evaluation of phage 6557 *Furthermore the strains* with unknown antibiotic resistance have been left out The remaining 568 strains have been divided according to antibiotic sensitivity 434 strains lysed by all the four experimental phages have been entered *in the bottom line of* Table 4 134 strains resistant to one or more of the experimental phages have been placed according to lytic response the outcome of which forms the basis for the final comparison of the suitability of the phages When arranged separately each phage type is seen to differ from the combined type lysed by all four phages and in different ways

TABLE 4

Comparison of the Distribution of Strains Lysed by One or More of the Experimental Phages According to Their Antibiotic Patterns

		Patterns of antibiotic resistance			
		Sensitive P PS or PT	*PST or* PSTC	*PSTT or* PSTCE	*Total*
Strains resistant to one *or more of the experi* mental phages but lysed by	D	30	17	7	54
	77Ad	14	42	16	72
	B5	21	27	21	69
	6557	25	13	15	53
Strains lysed by all four experimental phages		13	199	222	434

(NB! One strain may be registered several times according to its lytic pattern)

Taking the 134 strains lysed by only one two or three of the four experimental phages (i e the first four lines in *Table 4*) it can be estimated whether or not strains lysed by one of the phages differ from strains lysed by another phage in other words whether the strains characterized by one phage may *represent a random s lection* of the 134 strains or whether each of the phages seems to select a section of the material differing in antibiotic pattern from the section *selected by another phage*

Type 77Ad seems to differ from type D from type 6557 and probably from B5

Type D differs undoubtedly from type B5 but probably not from type 6557

The types B5 and 6557 do not seem to differ from each other and may be considered identical

Phage 77Ad lyses many strains with the antibiotic pattern PST(C) (42 out of 72 strains) Phage D defines an assemblage of strains often of low resistance (30 out of 54 strains) most of them resistant to penicillin only (20 out of 54 strains) only 7 out of these 54 strains had the pattern PST(C)E Phage 6557 has also a tendency to lyse strains of low resistance (25 out of 53 strains 12 of these being resistant to penicillin only) nevertheless it lyses a number of strains with the pattern PST(C)E (15 out of 53 strains)

Thus no very important differences between the suitability of the four phages as typing phages have been revealed

In order to determine the typing capacity of phage 6557 it has been used in the daily routine typing of staphylococci together with the conventional phages at Statens Seruminstitut since April 1st 1963 The number of strains tested from this date until December 31st 1964 amounts to about 24 000 about 17 per cent of which were non typable by means of the basic set Among these non typable strains about 54 per cent could be typed by phage 6557 About 98 per cent of strains lysed py phage 83A (RTD) and about 85 per cent of those lysed by one or more of the group III phages (RTD) are also lysed by phage 6557 whereas staphylococci of phage groups I and II are unaffected by the new phage

DISCUSSION

Each of the four phages examined D 77Ad B5 and 6557 can lyse many of the new epidemic NT staphylococci that show inhibition reactions with the phages normally lysing strains of group III Among the strains lysed by one of the four phages 74 per cent are also lysed by all the other three however the lytic abilities of the phages are not quite identical

As regards its capacity for lysing strains 6557 is neither superior nor inferior to the others

Using phage 6557 alone instead of the four phages tested in this investigation only 11 strains (5 per cent) out of 248 with the antibiotic pattern PST(C)E and 38 (15 per cent) out of 250 strains with the pattern PST(C) have been missed Out of the 70 strains of lower resistance (or fully sensitive) and of a more doubtful epidemiological significance as many as 32 (46 per cent) would have been missed by using phage 6557 alone

The overlapping of the phage reactions shows that the group of strains hitherto designated as NT is not homogeneous as regards phage

susceptibility This heterogenicity has been noticed by several authors (*Jevons & Parker* 1964 *Comtois* 1965 *Hurst* 1965) *Comtois* considered phages B5 and 77Ad as suitable typing phages either singly or in combination whereas he thought the phages D UC18 and H6 might be used for subgrouping *Smith et al* (1965) also proposed that the phages 77Ad and B5 should be included in the basic set and in 1966 The International Committee on Phage Typing of Staphylococci (Moscow Congress) included them under the designations 84 and 85 respectively *Kundsin et al* (1964) proposed that phage UC18 should be adopted in the basic set whereas *Hurst* (1965) found the phages UC18 and SF inadequate for typing

If only the phages to be used for typing might be selected on the basis on their number exclusively there would hardly be any disagreement about adopting more than one phage among those used for characterizing the topical staphylococci But the principles underlying the selection of typing phages may need to be reconsidered

The classic criteria for typing phages (*Fisk* 1942 *Wilson & Atkinson* 1945 *Williams & Rippon* 1952 *Williams et al* 1953) suitable for inclusion in the basic set were 1) they must have the same lytic effect on strains with a probable epidemic connection—in other words the susceptibility for the phages must be sufficiently stable in a given epidemic situation 2) on the other hand the lytic field of activity must not be so broad that too many irrelevant staphylococci are lysed and the purpose of phage typing is wasted The importance of avoiding too large and too heterogeneous groups of strains has been illustrated by *Jevons & Parker* (1964) they pointed out that if phage D in 1958 had been included in the basic set of typing phages instead of phage 83A the emergence of the new epidemic type as an individual type would have passed unnoticed

However such criteria are not adequate if the phage typing were designed not only to recognize the epidemic connection between individual strains but also to clarify the emergence the spread and the characteristic properties of the varying epidemic strains If this were the case the susceptibility to the phages should demarcate as clearly as possible a group of strains that are homogeneous as regards other known properties too Within such a homogeneous group there should be a chance of finding the susceptibility to phages correlated also to properties which might influence the epidemic or pathogenic characters

So far this principle has not been generally recognized Unfortunately the number of bacterial properties available for an estimation of the homogenicity of the strains is somewhat limited In the present investigation we have made use of the susceptibility to concentrated phages (1000 × RTD) of group III together with the antibiotic patterns

As the strains have been collected during a limited period and within

a limited geographical area they are not necessarily epidemiologically independent of one another which in this connection is no disadvantage

In view of all this it seems that phage 77Ad (Table 4) differs from the other ones moreover it can lyse 74 per cent of the strains lysed by all four phages and strains with the antibiotic pattern PST and also strains of type 42B/47C at 1000 × RTD In addition to the 74 per cent phage D lyses strains which are penicillin resistant only *i e* strains which might form a distinct unit If one of the phages D or 77Ad was chosen as typing phage the mentioned categories of strains (type D P only type 77Ad PST and possibly type 42B/47C) might pass unnoticed designated as type D or type 77Ad

The phages B5 and 6557 have so much in common that it seems impossible under the present circumstances to say which one is the more suitable

Consequently it is preferred to employ more than one phage because this would make it possible to observe the emergence of new strains with blocked susceptibility to one or more of the phages (dependent upon the degree of lysogenization)

In the first place this point of view is supported by previous experience of the importance of the blocking phenomena caused by lysogenization (*Lowbury & Hood* 1953 *Rountree* 1956 *Rountree & Asheshov* 1961 *Asheshov & Winkler* 1966) For example in Great Britain and Denmark it seems probable that the new NT strains or some of them have arisen from type 83A which has acquired a blocking prophage (*Jevons & Parker* 1964 *Bulow & Rosendal* 1964) It might be mentioned that type 83A has not been found to be the ancestor of the epidemic NT strains in U S A (*Hurst* 1965) and Canada (*Comtois* 1965), this shows that the same or related phage type within various epidemic areas does not necessarily mean the same strain

Secondly this point of view has been strongly supported by later experience (*Rosendal & Bulow* 1968) after the phages 77Ad and B5 (now designated 84 and 85 respectively) were included in the international set of typing phages phage 6557 was still applied to all staphylococcal strains in the routine typing at Statens Seruminstitut *This has provided information that seems to be of theoretical and epi*demiological importance suggesting that the types 84/85/6557 and 6557—both resistant to P S T and E—differ from each other since the latter includes rather many methicillin (M) resistant strains which most often are also resistant to chloramphenicol (C) whereas the former is never M resistant

An account of the staphylococci lysed by phage 6557 will be given in the following papers Later an attempt will be made to elucidate the consequences of a spread of blocking phages among populations of hospital staphylococci

SUMMARY

A new experimental phage 6557 has been isolated It belongs to serotype B and its lytic spectrum is reported and compared with the spectra of the three phages D 77Ad and B5 isolated elsewhere

These four phages have been applied to a large material of strains that were not typable by the conventional set of typing phages at RTD

The suitability of each of the phages has been estimated according to its ability to delimit categories of staphylococci which might be regarded as entities by virtue of their common properties such properties being antibiotic sensitivity and susceptibility to standard phages at 1000 × RTD and to pool phages at RTD and 1000 × RTD

The spectra of the four experimental phages were rather uniform although the sections of the NT material lysed by each of the phages were not identical Thus no important differences between the suitability of the four phages were revealed

Used in the routine laboratory phage 6557 was able to lyse 54 per cent of previously non typable strains Furthermore 98 per cent of strains lysed by phage 83A and 85 per cent of those lysed by one or more of the phages normally lysing group III staphylococci (RTD) were also lysed by phage 6557 whereas staphylococci of the groups I and II were unaffected by the new phage

Criteria for the selection of phages to be included in the typing set are discussed

It is concluded that in the study of the dynamics of the changes in staphylococcal populations it is an advantage to supplement the basic set with other new particular phages

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A NEW EPIDEMIC PHAGE TYPE OF *STAPHYLOCOCCUS AUREUS*

2 *Characteristics of Staphylococci Lysed by Phage 6557*[*]

By

P BULOW

Received 20 vii 67

In Part 1 of this study a new experimental phage 6557 was described It is the primary purpose of the present report by using a larger material to confirm the findings published in a preliminary report (*Bulow & Rosendal* 1964) viz that this phage is able to lyse strains characterized by being non typable multiple resistant to antibiotics lipase negative and resistant to mercuric chloride

Furthermore the properties of these strains are compared with those of strains lysed by other group III phages (incl 83A) as well i e the various types within the III 83A 6557 complex

These strains attract interest because they give us our first opportunity to follow the evolution of a new epidemic type since these strains—type 6557 (*Bulow & Rosendal* 1964) or atypical type 83A (*Jevons & Parker* 1964)—seem to be derived by lysogenic conversion from type 83A strains which in their turn in a corresponding way probably originate from group III strains

Some of these evolutionary problems have recently been subjected to investigations by *Jevons et al* (1966) who confirmed the findings by *Bulow & Rosendal* (1964) that the new type could have arisen from 83A by lysogenization with a prophage blocking the production of the *Tween splitting enzyme and the susceptibility to typing phage 83A* They did not succeed in demonstrating that other cultural characteristics apart from lemon yellow pigmentation could be changed by the presence of the blocking phage such as production of beta lysin and staphylokinase and resistance to neomycin and bacitracin as might be expected if naturally occurring strains of type 83A were compared with the new type

In the present and following papers the relationship between the types within the complex III 83A 6557 will be reviewed with special regard

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the prophage content of the strains In order to characterize the rains the examination includes resistance to mercuric chloride Tween 80 reaction and antibiotic sensitivity Special attention has been paid to the distinction between dissociated and double resistance to ery thromycin (*Garrod* 1957) Strains with the first mentioned type of re sistance are sensitive to oleandomycin consist of cells of various degrees of resistance to erythromycin and are influenced antagonistically by the two antibiotics *in vitro* (*Chabert* 1956) Strains of the double resitant type are resistant to oleandomycin as well as to erythromycin and all cells in a culture are equally resistant to high concentrations of both antibiotics This distinction seems appropriate in the characterization of strains since the dissociated resistance is an inducible property (*Pattee & Baldwin* 1962 *Weaver & Pattee* 1964) whereas the double resistance seems to depend on a constitutive enzyme system

MATERIALS AND METHODS

Two materials of phage typed *Staphylococcus aureus* strains have been available and the strains applied in the present investigations have been registered in one or both

The total material comprises about 73 000 strains and is described in Part 1 of this study

Strains included in this material

1799 strains type 83A/6557

1946 strains type 6557

4936 strains lysed by group III phages with or without additional lyses by phage 83A and/or 6557

250 non typable strains

The bacteraemia material comprises 1634 strains obtained from cases examined in the period from 1957 to 1965 Part of this material has been described previously by *Jessen et al* (1963) The antibiotic sensitivity of these strains has been checked in one single laboratory

Strains included in this material

231 strains type 6557

54 strains lysed by group III phages as well as phage 6557 but not by phage 83A

173 strains (type not considered) resistant to PST and PSTE

Furthermore 13 type 83A/6557 strains resistant to erythromycin have been col lected from both materials

The methods have been described in Part 1 of this study with the exception of the technique for demonstrating dissociated versus double" resistance in erythro mycin and resistance to neomycin and bacitracin

However resistance to erythromycin has for some strains been determined by a more refined method permitting a distinction between inducible (dissociated) and constitutive (double) type of resistance Two paper discs containing 10 μg of erythromycin and 50 μg of oleandomycin respectively have been used for preparing plates for the prediffusion test (*Thomsen* 1962)

The antagonism phenomenon (*Chabert* 1956) demonstrated in Fig 1 is characteristi of the inducible type of erythromycin resistance

Resistance to neomycin and bacitracin was determined by streaking one loop f l f and 18 hour broth culture onto nutrient agar plates containing 5 μg and 15 μr t r1 of neomycin and bacitracin respectively

The Reliability of the Estimation of Sensitivity to Erythromycin

As mentioned under Methods some of the sensitivity test em d h e been performed in different laboratories and as the resistance to tr r my n cems t play a particular role as a characteristic of type 6557 sp ial tt t n ha been

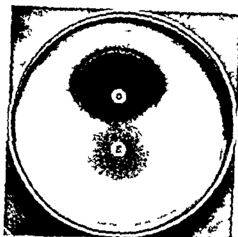


Fig 1

The dissociated or inducible erythromycin resistance O = oleandomycin E = erythromycin The appearance of the inhibition zones shows the "antagonism phenomenon characterizing the dissociated or inducible type of resistance to ertyhromycin (The plates have been flooded with an 18 hour broth culture and incubated at 3o C for 48 hours)

drawn to the reliability of the information concerning the sensitivity to this antibiotic

According to *Eriksen et al* (1960) the size of the inoculum as well as a prolonged incubation period (48 instead of 18 hours) is decisive if resistant cells present in the cultures are to be revealed

Table 1 shows a comparison between the results obtained in various laboratories all over Denmark and those found in one single laboratory[1] taking the above mentioned precautions in tests of resistance to erythromycin It is seen that 16 out of 79 strains previously found sensitive to erythromycin were resistant to erythromycin when re tested by the more refined procedure whereas only 2 out of 44 strains determined as erythromycin resistant were sensitive to erythromycin

TABLE 1

The Results of Sensitivity Tests of 123 Strains with the Antibiotic Patterns PST and PSTE from the Bacteraemia National Collected in 1955 and Studied in Several Laboratories all over Denmark as Compared with Those Studied in One Laboratory only

Antibiotic patterns estimated in several laboratories	Antibiotic patterns estimated in one laboratory only	
PST 79 strains	PST 63 strains	PSTE 16 strains (20%)
PSTE 44 strains	PST 2 strains (4 5%)	PSTE 42 strains

Thus it may be concluded that the figures to be obtained when the results from various laboratories are used represent minimum values

[1] Institute of Medical Microbiology University of Copenhagen (K. Bienertz) k sen M D Reader in Medical Microbiology)

The Reliability of the Estimation of Sensitivity to Some Other Antibiotics

The reliability of the statements received from different laboratories concerning the sensitivity to penicillin streptomycin and tetracyclines can be judged from Table 2

Table 2 includes 1799 type 83A/6557 strains in the total material collected during the years 1963 and 1964 A randomly selected section comprising 316 strains has been re examined and as appears from the Table the results do not disagree considerably from those obtained in other laboratories even if the probability of a more intimate epidemiological connection in the minor material and the limited numb r of strains are taken into account

TABLE 2

Results of Sensitivity Tests Performed in Various Laboratories as Compared with the Author s Re examinations of a Section of the Material Comprising Type 83A/6557 Strains from the Years 1963 and 1964

Type 83A/6557	% sensitive	Percentage of strains resistant to P	PS+PT	PST(C)	PSTE
1799 strains in the total material	4	10	4	76	6
316 strains casually selected for re examination	3	7	5	74	9

Thus it seems permissible to conclude that the information received from the different laboratories concerning the antibiotic sensitivity of the staphylococci forwarded to the phage typing laboratory is acceptable and may form the basis of the following studies of the antibiotic patterns of the main types within the phage type complex III 83A 6557

RESULTS

1 *Strains Lysed by Phage 6557 only (Type 6557)*

Antibiotic resistance Survey of the total material Previously it has been stated (*Bulow & Rosendal* 1964) that staphylococci lysed by phage 6557 alone were usually resistant to several antibiotics in contrast to those not lysed by any of the conventional phages

A comparison between the two categories has been summarized in Table 3 it shows that sensitivity to phage 6557 is correlated with multiple antibiotic resistance and that in particular the combined antibiotic pattern PST(C)E is common within type 6557 being shared by about 49 per cent of the strains

However two circumstances tend to bring about an unduly low estimated number of strains with this pattern and a too high percentage for strains with the combined pattern PST

In the first place as mentioned in Table 1 in routine sensitivity tests made in different laboratories resistance to erythromycin may pass unnoticed in some cases and the pattern be reported as PST instead of PSTE

Secondly the strains entered in Table 3 as type 6557 are not a homogeneous entity they include a number of strains that can be lysed

TABLE 3

Antibiotic Resistance on Non typable Strains. A Comparison between Strains Lysed by Phage 6557 and Strains Still Non typable

	Sens	Percentage of strains resistant to P	S	T	C	E
Strains lysed by phage 6557 at RTD (575)	1.5	97.0	94.0	93.[illegible]	13.8	48.7
Strains not lysed by any phages at all ("NT") (250)	40.0	53.7	21.2	19.8	1.4	4.6

	Percentage of strains with the antibiotic patterns only P	PS or PT	PST	PSTC	PSTE	PSTCE
Strains lysed by phage 6557 at RTD (575)	3.9	2.5	38.2	5.[illegible]	40.0	8.7
Strains not lysed by any phages at all ("NT") (250)	31.2	9.2	7.8	1.0	4.1	0

P = penicillin S = streptomycin T = tetracycline C = chloramphenicol
E = erythromycin

by phage 83A at 1000 × RTD and which more properly should be considered as type 83A strains or more correctly type 83A/6557 (see Part I, p. 151). The desirable distinction demanding the use of undiluted phage 83A is however inconvenient in a routine laboratory procedure and has not been made. Consequently too many strains with the resistance pattern PST typical of type 83A (see later) have been included under the designation type 6557.

If in spite of the probable heterogeneity strains lysed by phage 6557 at RTD but not by any other of the conventional typing phages at RTD are accepted as a distinct type, this would comprise a very high proportion of multiple resistant strains, in particular it would be far the most erythromycin resistant type known in Denmark (*Rosendal & Bülow* 1967). Table 3 shows that about 50 per cent are strains with the pattern *PSTE and 40 per cent strains* with the pattern PST (or if the figures are corrected according to Table 1 the percentages will be about 60 and 40, respectively).

Antibiotic resistance. Detailed examination of the selected material. The selected material of bacteraemia strains collected in the years 1963–1965 was studied separately and more extensively. It included *148* type 6557 strains which were examined *for antibiotic resistance to* P, S, T, C and E in one laboratory; attention was paid to the mentioned difficulties involved in erythromycin sensitivity *tests. The antibiotics* tested also included methicillin (M), neomycin (N) and bacitracin (B). In this material *resistance to the two last named antibiotics was always* combined and it was correlated to multiple resistance to the four or five

conventional antibiotics apparently in particular to erythromycin (see Table 4)

Out of 98 strains resistant to P S T and E (i e the patterns PSTE PSTCE PSTEM and PSTCEM) 81 strains (83 per cent) were also resistant to neomycin and bacitracin as opposed to 2 (5 per cent) of the 41 strains resistant to P S and T (i e PST PSTC and PSTM)

TABLE 4

Correlation between Neomycin and Bacitracin Resistance and Resistance to the Conventional Antibiotics

"Type 6557 strains in the bacteraemia material collected in the years 1963–1965

	Antibiotic patterns										
	Sens	P	PT	PST	PSTC	PSTM	PSTE	PSTCE	PSTEM	PSTCEM	Total
Total number of strains	2	5	2	36	3	2	67	22	6	3	148
Neomycin and bacitracin resistant	0	0	0	2	0	0	62	19	0	0	83 (56%)

Eleven out of 148 strains were methicillin resistant and none of these were N or B resistant (If the M resistant strains were omitted from the E resistant combined patterns the proportion of strains resistant to N and B would be 81 out of 89 (91 per cent instead of the above mentioned 83 per cent)) The incompatibility of M resistance with N and B resistance is probably a constant finding it will be commented on in a subsequent paper (*Rosendal & Bulow* 1968)

A total of 56 per cent of the type 6557 strains were resistant to neomycin and bacitracin

Mercury resistance and Tween 80 reaction A positive correlation between antibiotic resistance and resistance to mercuric chloride (Hg) has been demonstrated (*Moore* 1960 *Jessen et al* 1963) and a negative correlation between antibiotic resistance and the production of diffusible Tween 80 splitting enzyme (TW) (*Jessen et al* 1959 1963) The Hg and TW reactions of type 6557 strains have therefore been tabulated against the antibiotic resistance using a material of 1946 strains Among these strains 79 per cent were Tween negative (TW —) and 83 per cent mercury resistant (Hg +)

As seen from Table 5 the positive correlation between Hg resistance and multiple antibiotic resistance has been confirmed

The lack of demonstrable Tween 80 *splitting enzyme seems most* closely related to the resistance to streptomycin and possibly to erythromycin but more independent of the resistance to penicillin tetracyclines and chloramphenicol

2 *Strains Lysed by Phage 83A and 6557 (Type 83A/6557)*

As previously mentioned (Part 1 p 155) 98 per cent of strains lysed by the typing phage 83A are lysed also by phage 6557 hence the type designated as 83A/6557 is virtually identical with the type previously called 83A

TABLE 5

Percentage of Hg+ and TW— strains within Certain Groups or Types of Staphylococci Divided According to Antibiotic Patterns

Phage types	Total number of strains	Antibiotic patterns: Sens P and PT			PS			PST+PSTC			PSTE+PSTCE		
		% of total	% Hg+	% TW—	% of total	% Hg+	% TW—	% of total	% Hg+	% TW—	% of total	% Hg+	% TW—
6557	1946	17	20	29	2	87	73	37	95	83	44	98	97
83A/6557	1799	16	16	28	2	80	51	77	90	70	6	95	53
III"	4236	83	11	[illegible]	4	54	40	10	81	56	3	93	83

Type III" means strains lysed by one or more of the group III phages with or without additional lysis by the phages 83A and/or 6557
Hg+ = resistance to mercuric chloride TW — = Tween negative
Comprised in the total material 1963 and 1964

Antibiotic resistance About 77 per cent of 1799 strains isolated in 1963 and 1964 were reported to be resistant to P S and T including about 4 per cent that were resistant to chloramphenicol as well (Table 5) Only 6 per cent of the strains are also resistant to erythromycin The selected material of strains isolated from bacteraemia cases observed in the years 1963–65 included 100 strains of type 83A/6557 which were examined with regard to their resistance to antibiotics including M N and B as described on page 161 None of the 100 strains were found to be resistant to the three antibiotics However 4 type 83A/6557 strains among 750 in the total material of 1965 were found resistant to N and B (Table 7)

Mercury resistance and Tween 80 reaction A total of 62 per cent of the strains were TW negative and 79 per cent were Hg+

The ratio TW—/TW + is correlated with the antibiotic resistance but less simply than for the type 6557 strains (Table 5) The highest proportions of TW —strains (about 70 per cent) were found among strains with the patterns PST and PSTC whereas the percentage is lower (53 per cent) among strains that are also resistant to erythromycin

This type of strain follows the rules for positive correlation between Hg resistance and resistance to an increasing number of antibiotics (Table 5)

3 *Strains Lysed by one or more of Group III Phages, with or without Accompanying Lysis by the Phages 83A and/or 6557*

It has been stated earlier (Part 1 p 155) that about 85 per cent of strains lysed by one or more group III phages from the basic set at RTD are lysed also by phage 6557, constituting the types III/83A/6557 III/6557 III/83A (not lysed by phage 6557) and III (not lysed by any of the phages 83A and 6557) In the total material (1963-64) 4236 strains of the four mentioned phage patterns have been surveyed

Antibiotic resistance Most of the strains are only resistant to two antibiotics or less namely 83 per cent including about 18 per cent fully sensitive to the conventional antibiotics (60 per cent resistant to P only) Ten per cent of the strains have the pattern PST or PSTC the additional percentage of erythromycin resistant strains is as low as 3 per cent (Table 5)

Mercury resistance and Tween 80 reaction Among the group III strains collectively 30 per cent were TW—and 21 per cent were Hg+ Correlation between these properties and the antibiotic resistance will appear from Table 5

Phage patterns correlated to antibiotic resistance TW reaction and Hg resistance In order to show a possible correlation between the above mentioned characteristics and the sensitivity to the conventional typing phages of group III a smaller material of 357 strains received from one laboratory was examined

It was not found feasible to correlate the chosen properties to susceptibility to single typing phages as the various phage patterns were numerous

It was found that about 88 per cent of the strains were lysed by phage 6557 but not by 83A whereas about 50 per cent were lysed by both of these phages less than 2 per cent were lysed by phage 83A but not by 6557 If a single phage reaction is combined with susceptibility to phages 83A and/or 6557 it is most often due to phage 53 or phage 77

Thus the material can be divided into three subgroups by the aid of phages 83A and 6557 viz those with the patterns III/83A/6557 III/6557 and III (not lysed by any of the phages 83A or 6557) In Table 6 the two patterns III/6557 and III have been combined because a primary division did not show any difference between the properties of the strains within these two subgroups

However a conspicuous correlation is seen between susceptibility to phage 83A and antibiotic resistance TW reaction and Hg resistance (see Table 6)

Strains not lysed by phage 83A viz the type patterns III/6557 and III are resistant to more antibiotics than the strains of type III/83A/6557 are It is seen that 23 per cent of the strains in the first group and less than 1 per cent in the second group have the antibiotic pattern PSTE(C)

The percentage of TW— strains among the two groups III/6557 + III and III/83A/6557 is 56 and 31 respectively the percentage of Hg resistant strains is 42 and 11 respectively

These correlations confirm the concept that the two groups or types are different entities

It was true of all the recorded types belonging to the complex III 83A 6557 (i e 6557 83A/6557 III/6 57 + III and III/83A/6557) that no correlation was found between the TW reaction and the Hg resistance

TABLE 6

Correlation between Susceptibility to Phage 83A Antibiotic Patterns TW Reaction and Hg Resistance among Strains Lysed by One or More of the Group III Phages at RTD

		Antibiotic patterns			
		Sens P PS and PT	PST and PSTC	I STC and PSTCI	Total
III/83A/6557	TW—	50	6	0	56 (31[illegible])
	Hg+	13	6	1	20 (11%)
	Total number	171 (95%)	8 (4%)	1 (0 6%)	180
III/6557 and III"	TW—	50	11	38	99 (56%)
	Hg+	22	13	40	7[illegible] (4[illegible])
	Total number	123 (70%)	14 (8%)	40 (23%)	177

4 *Further Studies of the Erythromycin Resistance of Strains Belonging to the Phage-Type Complex III 83A 6557*

From the results reported here it seems that resistance to L N and B may be properties of significance for a tentative distinction between the types within the III 83A 6557 complex Therefore a selected material of 167 epidemiologically unrelated strains has been tested for type of erythromycin resistance (dissociated or double Garrod 1957) Furthermore resistance to N and B has been correlated to the inducible and constitutional forms of erythromycin resistance and resistance to oleandomycin

The results of this investigation appear from Table 7 It should be noted that the strains are divided into three antibiotic patterns

The first two antibiotic patterns belong to *Garrod* s dissociated type corresponding to the inducible form of resistance (*Weaver & Pattee* 1964) and the third belongs to the double type— or in *Karstrom* s terminology (1938) the constitutive form of resistance It appears that the erythromycin resistance typical of "type 6557 is due to an

inducible system namely in 93 per cent of the strains whereas the double or constitutive form of resistance seems fairly rare (7 per cent). Neomycin and bacitracin resistance is exclusively combined with the inducible E resistance which feature has also been found within the two other phage type patterns of Table 7. Double resistant strains are chiefly found within phage group III (here 31 per cent). Neomycin and/or bacitracin resistance has never been found among strains of the double resistant form, in other words oleandomycin resistance and neomycin/bacitracin resistance is never found within the same staphylococcal strain in this laboratory. This seems to be in clear contrast to the finding by *Jansson & Wager* (1962) who reported that 54 per cent of N resistant staphylococcal strains in Finland were also resistant to oleandomycin. But the two materials are probably not comparable as the latter was *collected in 1959–60 and seems to include group I as well as group II* staphylococci.

TABLE 7

The Relation between the Antibiotic Patterns and the Phage Types of the III 83A 6557 Complex with Special Reference to the Type of Erythromycin Resistance

Phage type	PSTE (inducible E resistance)	PSTENB (inducible E resistance)	PSTEO (constitutive E resistance)
6557 (100 strains)	7	*86*	7
83A/6557 (13 strains)	7	4	2
"III" or III/6557 (54 strains)	6	31	17

"III" means reaction with one or more of the group III typing phages.
P = resistance to penicillin. S = resistance to streptomycin. T = resistance to tetracyclines. E = resistance to erythromycin. N = resistance to neomycin. B = resistance to bacitracin. O = resistance to oleandomycin.

Eight strains among the types 6557 and III/6557 included in Table 7 were methicillin resistant; they were all sensitive to neomycin and bacitracin whereas five were oleandomycin resistant. Another remarkable fact is that all strains within the two latter phage types were TW— whereas five of the 13 type 83A/6557 strains were TW+, all of which had the inducible form of resistance. This will be discussed in a subsequent paper.

Furthermore a material of strains within the III 83A 6557 complex with the antibiotic pattern PST has been tested for sensitivity to oleandomycin, neomycin and bacitracin. The results were as follows: out of 60 strains of type 6557 none were O resistant while two strains were N and B resistant; out of 50 strains of type 83A/6557 none were N or B resistant but one was surprisingly found to be O resistant; out of 14 strains of type III/6557 3 were found to be B resistant but none were N or O resistant.

An analysis of the sensitivity to the typing phages of group III strains with the antibiotic patterns shown in Table 7 has revealed some interesting features None of the strains recorded are lysed by phage 83A and the basic pattern is 7/47/53/54/75/77/6557 which is often seen among strains of the inducible type of resistance to erythromycin (PSTE and PSTENB) but one of the lytic reactions may be blocked with the exception of lysis by the typing phages 53 and/or 77 Out of the 31 strains with the antibiotic pattern PSTENB 10 are lysed by phage 53 at RTD 13 are lysed by phage 77 at RTD with or without additional reaction with phage 6557 (at RTD or 1000 × RTD) and the remaining 8 strains are of the basic pattern with a possible blocking of a single lytic reaction In other words all the strains are sensitive to phage 53 and/or 77 at RTD This is in contrast to the 17 strains which belong to the double resistant form (PSTEO) these are all resistant to phage 53 (and often —10 strains— to phage 75)

DISCUSSION

The results confirm the conception previously advocated by *Bulow & Rosendal* (1964) that phage 6557 is suitable as an additional typing phage because it is able to lyse the great majority of non typable strains characterized by having other properties in common thus separating a group of multiple resistant strains from those less resistant (*Bulow* 1968)

These multiple resistant strains have probably both in Denmark (type 6557) and in England (atypical 83A *Jevons & Parker* 1964) arisen from 83A staphylococci by lysogenic conversion with a prophage blocking the sensitivity to typing phage 83A as well as the production of the Tween 80 splitting enzyme The properties of English and Danish strains of the new type seem to be similar they are lysed by the same experimental phages (*Jevons & Parker* 1964 *Bulow* 1968) and are characteristic in that they are multiple resistant (PST(C)ENB) TW — and Hg+ But the type 83A strains isolated in the two countries differ with respect to resistance to erythromycin 6 per cent being resistant in Denmark as opposed to 68 per cent in England (*Jevons et al* 1966) The same authors also found a percentage of TW + strains that was much higher (96 per cent) in their material than the percentage reported by the Danish authors (19 per cent *Rosendal & Jessen* 1964)

However in recent years erythromycin resistance of the Danish 83A strains has become much more frequent and among the resistant strains the percentage of TW+ strains ranges at about 50 Preliminary experiments later to be published (*Bulow*) seem to indicate that resistance to erythromycin and Tween positivity may be dependent upon properties of prophages thus being subject to variations

As staphylococcal phages have played an important role in the evolution of the new epidemic type some problems arise First do the phages spread epidemiologically among hospital staphylococci or does a newly lysogenized strain spread among hospital patients? Second are the phages responsible for the new properties of the host cells which make them more fit to survive and spread in the hospital milieu?

The answer to the first question is probably a yes and no It is common knowledge (*Williams et al* 1960) that a particular staphylo

coccal strain with certain epidemic properties (thus also the new epidemic type) is able to spread among patients but epidemic spread of staphylococcal phages in a population of hospital staphylococci has never been demonstrated

Some of the results discussed in the present paper suggest that phages capable of blocking the susceptibility to phage 83A might be present both in type 6557 and in other group III staphylococci as resistance to phage 83A is correlated with resistance to erythromycin neomycin and bacitracin

If it were possible to demonstrate identical phages in epidemiologically unrelated multiple resistant strains of these types it might be permissible to presume an epidemic spread of one special phage among hospital staphylococci and the two evolutionary processes

1) 83A/6557 → 6557
2) III/83A/6557 → III/6557

might be explained as the outcome of this

At the present moment it is not possible to answer the second question either It is impossible to tell whether resistance to erythromycin neomycin and bacitracin properties characterizing the lysogenized types in the right column are phage dependent *Jevons et al* (1966) failed to show whether the mutation rate to resistance to neomycin increased in lysogenized cultures of 83A strain if compared with the parent strain

It does not seem likely that staphylococci are able to acquire resistance to other antibiotics either as a direct result of lysogenization although a few reports on this topic have appeared Some confusion seems to have existed about the concepts lysogenization and transduction Thus *Cavallo & Terranova* (1955) claim that development of resistance to streptomycin can be caused by lysogenization of staphylococci but mention *that the phenomenon observed is a transduction* and as a matter of fact it cannot be denied that an increase of the mutation rate possibly caused by lysogenization may have taken place

Blair & Carr (1961) describe gain as well as loss of resistance to penicillin as a cause of lysogenization However according to reports published during recent years penicillinase production has apparently been proved to be a transducible property (*Ritz & Baldwin* 1958) the production of this enzyme being controlled by extrachromosomal plasmid(s) (*Novick* 1963 *Harmon & Baldwin* 1964 *Novick & Richmond* 1965) which are lost from the bacterial cell with a frequency of 10^{-3} in all staphylococcal cultures (*Novick* 1963) *Blair & Carr*s results may probably be explained as transduction of plasmids and — where it is loss of resistance—as a loss of these plasmids even if the latter phenomenon might be a result of lysogenization (by analogy with the phenomenon called prophage substitution) They discuss their results in relation to those obtained by *Morse* (1959) who transduced resistance to streptomycin and novobiocin and to those obtained by *Comtois*

(1960) who did not succeed in changing the antibiotic resistance as a result of lysogenization of type 80/81 strains.

Since *Ritz & Baldwin* in 1958 demonstrated that the ability to produce penicillinase was a transducible property among *Staphylococcus aureus* strains, several reports dealing with transduction of resistance to various antibiotics within this group of organisms have been published. A survey of the literature from recent years and the kind of antibiotic markers used are given in Table 8.

TABLE 8

Survey of Literature Dealing with Transduction of Antibiotic Resistance between Strains of Staphylococcus aureus

Antibiotic resistance marker	References
Penicillin	*Ritz & Baldwin* 1958
Streptomycin, novobiocin	*Morse* 1959
Tetracycline	*Mitsuhashi et al* 1961
Penicillin, tetracycline, novobiocin	*Pattee & Baldwin* 1961
Tetracycline	*Collins & McDonald* 1962
Streptomycin, novobiocin	*Dowell & Rosenblum* 1962 (a and b)
Streptomycin	*Korman & Berman* 1962
Erythromycin	*Mitsuhashi et al* 1962
Erythromycin, oleandomycin	*Pattee & Baldwin* 1962
Chloramphenicol, novobiocin	*Collins & Roy* 1963
Tetracycline, erythromycin	*Kuwahara et al* 1963
Tetracycline	*Nuwa et al* 1963
Penicillin (plasmid)	*Novick* 1963
Streptomycin	*von Graevenitz* 1964
Penicillin (+ Hg)	*Richmond & John* 1964
Tetracycline (*in vivo*)	*Jarolmen et al* 1965
Tetracycline	*Mitsuhashi et al* 1965
Penicillin, tetracycline	*McDonald* 1966

It seems beyond doubt that transduction of resistance markers among staphylococci is a general phenomenon. Transduction *in vivo* was first demonstrated by *Velaudapillai* (1960) who studied *Salmonella* infections in chick embryos and mice. In 1965 *Jarolmen et al* were able to transduce resistance to tetracycline to staphylococci *in vivo* by intravenous injection of staphylococcal phages propagated on tetracycline resistant strains following previous injection of sensitive staphylococci. If tetracycline were administered the injected staphylococcal cells would change more readily from sensitive to resistant.

These results may help to clarify the development of antibiotic resistance among hospital staphylococci, which may be the result of transduction of resistance markers together with a selective pressure of the antibiotics administered. However, it is still possible that the high mutation rate among staphylococci may be a sufficient explanation of the emergence of resistance when the use of antibiotics is involved.

In the light of these considerations future investigations will follow

two lines the consumption of antibiotics in selected hospitals will be correlated with the emergence and spread of the new multiple resistant type within these hospitals Furthermore *in vitro* experiments with transduction of resistance to erythromycin among staphylococci will be performed

SUMMARY

An experimental phage 6557 lysing a majority of non typable epidemic *Staphylococcus aureus* strains is found to be suitable as an additional typing phage

The properties of strains lysed by the experimental phage are described

Strains lysed by phage 6557 alone are characteristic in that they are multiple resistant Tween negative and resistant to mercuric chloride more frequently than the other strains investigated

Resistance to erythromycin neomycin bacitracin and methicillin is less frequently encountered in strains lysed by typing phage 83A than in strains resistant to this phage i e strains lysed by phage 6557 alone or in combination with other group III phages

Probably the same prophage can block the susceptibility to phage 83A in all strains investigated It is discussed whether the development of resistance to erythromycin neomycin and bacitracin also is phage dependent and favoured by consumption of the antibiotics in question

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BRIEF REPORT

ABSORPTION FROM THE PERICARDIUM

By *Trond Kluge*

Absorption of tracer substances from the pericardial cavity in rats was studied by electron microscopy lymphography and analysis of thoracic duct lymph

Thorium dioxide (Thorotrast) was absorbed in the form of free particles by pinocytosis and was incorporated into electron dense bodies interpreted as lysosomes Thorium particles were not observed in the intercellular spaces Absorption was more rapid through the parietal than through the visceral mesothelium The visceral cells containing an abundant endoplasmic reticulum did not participate in the absorption

Horseradish peroxidase a protein of MW about 40 000 was mainly absorbed through the intercellular spaces Small amounts of reaction product were observed inside cytoplasmic vacuoles and electron dense bodies The passage of protein molecules between mesothelial cells indicates that the tight junctions (zonulae occludentes) do not as previously suggested (1) seal off the intercellular spaces Similar observations on endothelium have recently been reported (2)

By lymphography drainage of thorium dioxide through the parasternal mediastinal and diaphragmatic lymphatics was observed Analyses of thoracic duct lymph indicated that lymph from the pericardium entered the duct in its cranial as well as in its caudal portion

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ASBESTOS BODIES IN THE LUNGS AND MESOTHELIOMA

A Retrospective Examination of a Ten Year Autopsy Material

By

I HAGERSTRAND L MEURMAN and B ODLUND

Received 29 xi 67

Since the thirties when pulmonary carcinomas in patients with asbestosis were reported (6 11) asbestos has been suspected to be carcinogenic In recent years the question of a possible relationship between exposure to asbestos and mesothelioma of the pleura as well as the peritoneum has attracted interest (20) We therefore considered it of value to study the autopsy material of mesothelioma at the Pathological Department General Hospital Malmo Sweden with regard to the presence of asbestos bodies Similar studies have been reported from several other places (5 8 10 12 14 16 17 21 23) In order to throw further light upon the problem of asbestos and mesothelioma we investigated the frequency of asbestosis in our autopsy material as well as the presence of asbestos bodies in the lungs in autopsy cases in an occupational group said to be exposed to asbestos (electricians)

MATERIALS AND METHODS

The material is derived from autopsies during 1957–1966 at the Pathological Department General Hospital Malmo Sweden Malmo is a town with about 250 000 inhabitants situated on the southern coast of Sweden Workers within the textile and shipbuilding industries and insulation workers are presumably the ones most heavily exposed to asbestos in Malmo The town has only one hospital with one pathological department where post mortem examinations are performed on about 98 per cent of patients dying in the hospital corresponding to more than 60 per cent of deaths in the town The total number of autopsies during this ten year period was 1? 763 The distribution with regard to the different years as well as the sexual distribution are shown in Table 1 Ten per cent of the material consisted of children under 15 years of age In every case of malignant tumour routine examination was made i lymph nodes from various sites and from lungs liver spleen kidney and the prostate in addition to the primary tumour and the macroscopically visible m t tases A total of 25 pleural mesatheliomata were diagnosed during this per od and in addition 2 mesotheliomata in which cases it could not be decided whether the tumou had started in the pleura or the pericardium (these two cases w in luded among the pleural mesotheliomata) as well as 8 mesotheliomata of the peritoneum (see Table ?) The criteria for a diagnosis of mesothelioma were strict A complete and thorough post mortem examination where no other prima tumour could be demonstrated was required The histology in each case vas well c nsistent with that of mesothelioma.

TABLE 1

The Distribution of Mesothelioma in the Entire Autopsy Material with Regard to Year and Sex

Year	Males	Females	Males under 15 years	Females under 15 years	Total	Pleural meso thelioma	Peritoneal meso thelioma
1957	46?	472	53	47	938	4	0
1958	451	487	59	51	938	2	4
1959	547	547	59	64	1094	3	0
1960	617	577	69	48	1194	2	0
1961	521	699	68	58	1220	2	1
1962	730	590	64	49	1320	4	1
1963	709	699	49	51	1408	1	0
1964	716	748	61	53	1464	5	2
1965	793	7?9	74	5?	15??	2	0
1966	819	846	90	61	1?6?	2	0
Total	63?9	6394	??1	539	1??63	27	8

The investigation included the following items

1 *Every case of mesothelioma* was re assessed the post mortem record was *reviewed and the histology re examined*

2 Thirty four mesotheliomata (*in one case of pleural mesothelioma* no blocks of lung tissue were left) *were examined for the presence of asbestos bodies in lungs* – The control series comprised 34 autopsy cases of matching age and sex chosen at *random excluding cases of pulmonary tumours (see Table 3)*

3 Since there was no diagnosis of asbestosis in the entire autopsy material during *the years 1957–1964 we reviewed this material to see whether the disease might have* been overlooked. We re assessed every post mortem record by re examining the short *clinical history that was available as well as the pathological anatomical diagnosi* and the macro and micros opic description of lungs and pleurae *It was suspected that 15 cases of uncertain lung changes* expecially pulmonary fibrosis might have been asbestosis The lung sections from these 15 cases were re examined

During 1965 and 1966 when this investigation was going on we followed the daily autopsy work with special attention to asbestosis

4 *Lung* sections from 23 electricians included in the autopsy material from the years 1957 1964 were examined for the presence of asbestos bodies Electricians were the only ones who by way of their profession were exposed to asbestos among whom we could get enough cases in the autopsy material, since the occupation was not usually noted

In every case examined for asbestos bodies two sections from healthy lung tissue 30 microns thick and unstained were used The criteria for asbestos bodies were the same as those set up in the classical description by *Beger* (1) All bodies visible in ordinary light microscope were counted regardless of their length See Figures 1 and 2 The countings were made by one investigator (*Meurman*)

Figs 1–4

Fig 1 Asbestos body (Autopsy No 48 (4) × 1600

Fig 2 Asbestos body (Autopsy No 740/64) × 1?00

Fig 3 Macrophoto of peritoneal mesothelioma with grape like growth (Autop No 232 58)

Fig 4 Microphoto of peritoneal mesothelium (Autopsy No 486 58) Abdominal muscle at bottom of picture Hx–e × 75


2
3

TABLF
Anamnestic and Patholog cal Data

Year	Autopsy no	Age at death	Sex	Occupation	Site of primary tumour	Lymph node metastases
Pleural mesotheliomata						
57	154	40	♂	watch maker	lt pleura	mediastinum
57	285	58	♂	engineer (foreman of shipyard)	rt pleura	
57	510	67	♀	house wife	pericard pleurae	mediastinum
57	665	82	♀	house wife	lt pleura	mediastinum
58	402	60	♀	hospital nurse	rt pleura	
58	854	69	♂	police officer	rt pleura	
59	186	52	♂	dealer (frame manufacturing)	rt pleura	mediastinum neck para aortal mesenterium rt ingue rt axilla
59	531	73	♂	stevedore	pericard rt pleura	
59	593	66	♀	shop assistant	rt pleura	para aortal mesenteriu mediastinum
60	317	67	♂	sugar mill worker	rt pleura	mediastinum
60	321	60	♂	electric fitter	rt pleura	
61	1004	73	♂	cabinet maker	lt pleura	
61	1089	74	♂	plater iron worker	rt pleura	
62	67	79	♂	shipyard worker	lt pleura	
62	340	72	♂	sugar mill worker	pleurae	
62	601	71	♂	housing agent	rt pleura	mediastinum rt and lt fossa supraclav
62	966	70	♀	widow	rt pleura	hilus para aortal
63	572	78	♀	house wife	rt pleura	mediastinum rt and lt fossa supraclav rt axill
64	48	60	♂	railway plater	rt pleura	
64	281	76	♂	railway filer	rt pleura	mediastinum

2
about the Mesothelioma Material

Other distant metastases	Number of asbestos bodies in lungs	Histological pattern epithelial	sarcomatous
rt lung liver pancreas kidneys lt adrenal brain vertebrae	not investigated	+	
lt pleura lt lung liver kidneys peritoneum	2	+	
	0	+ (psammoma bodies)	
rt pleura rt lung rt breast liver adrenals myocard skin	0	+	+ (in vertebral metastases)
	1	+	+
	0	+	
lungs liver kidneys vertebrae r ritoneum	1	+	+ (in vertebral metastases)
lung liver rt kidney vertebrae	8	+	+ (in vertebral metastases)
pleura peritoneum	0	+ (psammoma bodies)	
	1	+	
yocardium	>100	+	+
	2	+	+
pleura rt lung liver ertebrae	9	+	+ (in vertebral metastases)
	0		+
iver peritoneum	40	+	
	4	+	+
t pleura peritoneum	0	+	
pleura lungs vertebrae	0	+ (also vertebral metastases)	
iver vertebrae	>100	+	+ (in vertebral metastases)
	35		+

TABLE

Year	Autopsy no	Age at death	Sex	Occupation	Site of primary tumour	Lymph node metastases
64	*545*	*66*	♂	sanitary inspector	lt pleura	
64	786	68	♂	*workshop assistant*	*rt pleura*	mediastinum axillas lt fossa supraclav
64	1136	84	♀	hous wife	lt pleura	mediastinum axillas *fossae supraclav* para aortal
65	*117*	*72*	♂	*engine driver*	*rt pleura*	mediastinum para aortal, rt fossa supraclav
65	945	73	♂	instructor in textile *factory*	rt pleura	mediastinum fossae supraclav
66	308	72	♀	unknown	lt pleura	*mediastinum para aortal*
66	633	53	♀	charwoman	lt pleura	
Peritoneal mesotheliomata						
58	*232*	*65*	♂	*pipe layer*	peritoneum	
58	286	57	♀	house wife	*peritoneum*	
58	477	63	♀	accountant	peritoneum	mesenterium mediastinum
58	*486*	*80*	♂	*carpenter*	*peritoneum*	*para aortal*
61	161	65	♂	engine driver	peritoneum	*rt ingue*
62	148	45	♀	mender	peritoneum	
64	177	83	♀	cook	peritoneum	
64	*740*	*78*	♂	stock room man	peritoneum	

RESULTS

In the following report of the results the same figures as above will be used to denote the detail studies

1 Significant data that emerged from the review of the meso thelioma material are summarized in Table 2 Every pleural meso thelioma grew as thick greyish white tumour masses encasing the entire lung or parts of it (the upper lobe) and obliterating the pleural cavity The tumour grew into interlobar as well as interlobular septa In many cases the tumour was macroscopically mucoid The pleural mesotheliomata showed a marked tendency to infiltrating growth into the thoracic wall pericardium mediastinum and through the dia phragma into the peritoneum or retroperitoneally around the adrenals In addition in 15 cases there were metastases to lymph nodes and in 18 cases metastases to other organs (in 12 cases to the lungs and or the

2 (cont.)

Other distant metastases	Number of asbestos bodies in lungs	Histological pattern epithelial	sarcomatous
	0	+	
lt pleura lungs peritoneum	5	+	
peritoneum lt m psoas	0	+	
lt pleura lt lung liver	0	+	
lungs liver peritoneum myocardium	0	+	
	5	+	
lt kidney	2	+	
liver	9	+	
liver	0	+	
rt pleura	0	+	
liver	0	+	
	0	+	
	26	+	+
	0	+	
	>100	+	

other pleura in 11 cases to the liver in 9 cases to the peritoneum in 7 cases to bone and in 5 cases to the kidneys) All the metastases registered in the table represent true metastases not direct overgrowth

Occasionally the mesotheliomata of the peritoneum (3 cases) had the same macroscopic appearance as those of the pleura growing as greyish white masses on the parietal peritoneum as well as on the surfaces of abdominal organs In 5 cases the peritoneal mesothelioma grew in numerous greyish blue polyps or grape like formations ranging from a few mm to about 5 cm in diameter on the parietal as well as on visceral peritoneum (see Figure 3) The tumour was often mucoid The mesotheliomata of the peritoneum did not metastasize as extensively as those of the pleura either to lymph nodes or to other organs

The histological appearance of the peritoneal mesotheliomata was of epithelial character (see Figure 4) sarcomatous features were noted

TABLE 3

Anamnestic and Pathological Data about the Control Series

Year	Autopsy no	Age at death	Sex	Occupation	Number of asbestos bodies in lungs	Main disease
Controls matching patients with pleural mesothelioma						
57	305	55	♂	sea captain	1	*Cancer ventriculi*
57	532	67	♀	house wife	0	*Cancer ventriculi*
57	683	84	♀	house wife	2	*Cancer oesophagi*
58	416	60	♀	hospital nurse shopassistent house maid	1	*Intoxicatio barbiturica*
58	865	69	♂	engineer	0	*Infarctus cordis*
59	199	56	♂	bank cashier	1	*Reticular cell sarcoma*
59	541	71	♂	headmaster	0	*Chronic pyelonephritis*
59	599	67	♀	gymnastics teacher	0	*Lung tuberculosis*
60	337	65	♂	travelling agent	0	*Cancer pancreatis*
60	339	65	♂	railway official	0	*Cancer vesicae urinariae*
61	1008	74	♂	night watchman	1	*Lymphatic leukemia*
61	1124	74	♂	merchant	0	*Infarctus cordis*
62	73	77	♂	book binder	0	*Cancer oesophagi*
62	342	73	♂	salesman	0	*Giant cell arteritis*
62	63[illegible]	67	♂	stock room man	0	*Infarctus cordis*
62	975	70	♀	house wife (husband electric fitter)	0	*Cancer ventriculi*
6[illegible]	573	78	♀	house wife	1	*Cancer ventriculi*
64	52	73	♂	turner	5	*Cirrhosis et cancer hepatis*
64	283	73	♂	work shop master	0	*Diabetes mellitus + Thrombosis a ren dx*
64	578	69	♂	colour sergeant	0	*Infarctus cordis*
64	807	71	♂	police constable	0	*Infarctus cordis*
64	1100	8[illegible]	♀	house wife	0	*Embolia pulm*
65	120	[illegible]	♂	stoker care taker	2	*Infarctus cordi*
65	952	68	♂	painter	5	*Encephalomalacia*
66	31[illegible]	74	♀	unknown	0	*Carcinoid*
66	691	64	♀	stock room woman (later house wife)	1	*Cancer colli uteri*
Controls matching patients with peritoneal mesothelioma						
58	243	68	♂	smith	0	*Cancer vesicae urinariae*
58	307	57	♀	cook	0	*Malignant glioma*
58	493	65	♀	house wife	[illegible]	*Multiple myeloma*
58	488	82	♂	travelling inspector	0	*Cancer coli*
61	164	[illegible]9	♂	plater	[illegible]0	*Subarachnoid haemorrhage*
6[illegible]	346	47	♀	unknown	0	*Cardiac arrest during anasthesia*
64	180	87	♀	unknown	0	*Ruptura aneurysma aortae*
64	753	[illegible]0	♂	registration clerk	0	*Infarctus cordis cancer coli cancer prostata*

only in one case Among the pleural mesotheliomata only 2 were of purely sarcomatous appearance but in addition sarcomatous features were observed in 9 among the 27 cases Vertebral metastases especially tended to grow in a sarcomatous pattern (see *Figure 5*) but in one case



Figs 5–6

Fig 5 Vertebral metastasis of pleural mesothelioma (Autopsy No 10 9 61) with sarcomatous growth Htx-e × 168

Fig 6 Pleural mesothelioma with psammoma bodies (Autop No 593/59) Htx-e × 176

there were epithelial structure in a vertebral metastasis as well. In two of the pleural mesotheliomata, psammoma bodies were present (see Figure 6)

As the entire autopsy material included 12 763 cases the frequency of mesothelioma was 0.27 per cent 0.21 per cent of this were pleural and 0.06 per cent peritoneal. Figures will be slightly different if children under 15 years of age are excluded—0.3 0.23 and 0.07 per cent respectively

2 Asbestos bodies were found in 15 of the 26 cases of pleural mesothelioma mostly a few bodies were found but in 4 cases, bodies were numerous i e more than 20 No cases showed pulmonary fibrosis In the control series asbestos bodies were found in 10 cases but never in large numbers

Asbestos bodies were found in 3 of the 8 cases of peritoneal mesothelioma in 2 cases bodies were numerous There was no pulmonary fibrosis In the control series one case had many asbestos bodies one case had two

3 Among the 15 cases suspected of asbestosis only one case showed advanced pulmonary fibrosis and numerous asbestos bodies (autopsy No 123/60) This patient was an electric fitter who died at 54 from a metastasizing adenocarcinoma of pulmonary origin During 1965 and 1966 while this investigation was going on 2 cases of insignificant asbestosis were encountered One case (autopsy No 613/65) died from pronounced coronary sclerosis This 53 year old man who had been an insulation worker for 34 years also had apical caseocavernous pulmonary tuberculosis The other case was a 65 year old insulation worker who died from carcinoma of the urinary bladder (autopsy No 597/65)

4 Among the 23 electricians asbestos bodies were found in 18 cases in 17 of these only few bodies were seen whereas 1 case showed 37 bodies

DISCUSSION

All studies of mesothelioma suffer from a certain degree of uncertainty owing to diagnostic difficulties The cases of mesothelioma presented here fulfil the criteria generally required for this diagnosis in that no other primary tumour was found in spite of a careful post mortem examination and also in that the microscopic mode of growth and the histologic appearance were typical (13) Chu g et al (3) demand that the tumour preferably should not have metastasized but if it had then only to regional lymph nodes This criterion was fulfilled only in 8 among our cases of pleural mesothelioma

We should like to stress the fact that peritoneal mesothelioma often tends to grow as polypoid grape like greyish blue and haemorrhagic tumours This finding has previously been described by Cicero In der (4) and others

As to the histological pattern of mesothelioma we should like to point out a few details only Our cases confirm Hourihane s observation that vertebral metastases from pleural mesothelioma tend to grow in a sarcomatous pattern It has been claimed (8) that the presence of psammoma bodies in a tumour makes a diagnosis of peritoneal mesothelioma unlikely In this connexion it is of interest to observe that psammoma bodies were present in 2 cases in our material of pleural mesothelioma

It is difficult to compare the frequency of mesothelioma in our material with that in other autopsy materials owing to differences in their composition As mentioned above in our material the frequency of mesothelioma was about 0 27 per cent 0 21 per cent of which were pleural and 0 06 per cent peritoneal *Konig* (10) reported a frequency of pleural mesothelioma of 0 7 per cent and of peritoneal mesothelioma of 0 16 per cent (13 307 consecutive autopsies but no cases of asbestosis from the St Georg Hospital Hamburg)

From Dresden *Rotzsch* (16) reported 25 pleural and 2 pericardial mesotheliomata among 21 651 autopsies (0 12 per cent)

In the Bernhard Baron Institute of Pathology London (9) the frequency of mesothelioma during 1950–1963 was found to be 0 3 per cent *Vigliani* (23) found pleural mesothelioma in 0 3 per cent in an autopsy material comprising 24 700 individuals over 40 years of age from Turin Milan and Pavia The frequency in our material is thus in fairly good agreement with findings in previously reported materials

Considerably higher frequencies of mesothelioma have been reported from autopsy materials consisting of cases of asbestosis or cases exposed to asbestos *Vigliani* (23) discovered 3 mesotheliomata among 172 autopsy cases with asbestosis (1 7 per cent) *Selikoff* (17) found 10 mesotheliomata in an autopsy material comprising 307 asbestos insulation workers in New York and New Jersey (3 2 per cent) In our mesothelioma material there was no case of asbestosis and in the entire autopsy material from the years 1957–1966 only one case of true asbestosis could be demonstrated

Asbestos bodies were encountered in 15 among 26 cases of pleural mesothelioma and in 10 cases in the matching control series If Churg s strict criteria for the diagnosis of mesothelioma were observed and our metastasizing mesotheliomata of the pleura thus excluded 8 cases remained 5 of which showed asbestos bodies Bodies were numerous only in one of these Asbestos bodies were encountered in 3 among the 8 cases of peritoneal mesothelioma in 2 cases in the control group The high frequency of malignant tumours in the control series may be criticized since malignancies other than mesothelioma also have been said to be related to asbestos exposure (7) It has not been possible to examine the material with regard to exposure to asbestos during life but in most cases occupations were known Cases with abundant asbestos bodies had had an occupation in which a certain exposure to

asbestos may well have occurred According to our opinion the most important difference between the mesothelioma and the control series seems to be that cases with abundant asbestos bodies were mainly found in the mesothelioma series (6 against 1 in the control series 20 asbestos bodies being taken as limit value) It is remarkable however that asbestos bodies were numerous only in one of the electricians who by way of their profession are said to be exposed to asbestos A possible explanation of this may be that asbestos is so closely attached to the insulating material that no dust occurs At any rate there was a higher percentage of cases with asbestos bodies among the electricians than among individuals with pleural and peritoneal mesotheliomata (78 per cent against 57 per cent respectively 33 per cent) The average age of patients included in the mesothelioma material and that of electricians was approximatively the same but there were 9 women among the patients with pleural mesothelioma 4 among the patients with peritoneal mesothelioma

Several studies of the presence of asbestos bodies in lungs in cases of mesothelioma have been published since 1960 when *Wagner* (21) pointed to a possible connexion between this tumour and exposure to asbestos It deserves to be mentioned that as early as in 1953 *Weiss* (22) Germany reported the presence of asbestosis in three cases of pleural mesothelioma and suggested a connexion between the two diseases Most investigations into the occurrence of asbestos bodies in the lungs of patients with mesothelioma indicate a marked positive correlation and thus differ from the material presented here Among 45 pleural mesotheliomata reported from Belfast (5) asbestos bodies were seen in 34 cases (75 per cent) whereas only 25 per cent in the control series had asbestos bodies *Owen* (15) Liverpool observed asbestos bodies in 7 out of 10 cases of mesothelioma in contrast to a 9 per cent frequency of asbestos bodies in 200 consecutive autopsies From London *Hourihane* (9) reported that asbestos bodies and/or asbestos fibres were present in 6 out of 7 definite pleural mesotheliomata and in 7 out of 11 definite peritoneal mesotheliomata where is neither asbestos bodies nor asbestos fibres were observed in 50 consecutive autopsies excluding cases of thoracic tumours On the other hand in studies in which the presence of asbestos bodies in lungs has been taken as starting point it has not been possible to demonstrate positive correlation between the occurrence of asbestos bodies and mesothelioma Among 100 autopsies in Pittsburg *Cauna* (1) found asbestos bodies in 41 cases none of which had mesothelioma Even more remarkable is *Meurman's* report (14) from the area surrounding the asbestos mine at Paakkila in Finland He examined 264 cases and demonstrated asbestos bodies in 57 6 per cent of these but he found no case of mesothelioma

It is very difficult to draw any conclusions from our own investigation as well as from the other investigations related above From our



7

Fig 7
So called pseudoasbestos body
Carbon needle covered with
segmented protein layer
(Autopsy No 740/64) × 1200

work and from earlier observations by *Meurman* (14) as well as by *Thomson* (20) it can be concluded that a small amount of asbestos bodies is a normal finding in adult individuals *Thomson* (20) studied 500 consecutive autopsies performed in Cape Town South Africa and 500 performed in Miami Florida using smears from the lung bases He found about the same frequency of asbestos bodies in the two materials i e 30 per cent among males 20 per cent among females In 85 per cent of the positive cases bodies were few In our opinion investigations into the frequency of asbestos bodies should be based on identical criteria and if possible performed by one and the same investigator Since a high percentage of asbestos bodies is normally seen in lungs the number of bodies must be much higher in cases with mesothelioma than in the controls if a positive correlation between asbestos bodies and mesothelioma is to be proved Among our 34 cases of mesothelioma 6 were found to have an abundance of bodies as compared with one case with numerous bodies among the controls

The presence of asbestos bodies is not equivalent to exposure to asbestos since other materials as well may give rise to a development of such ironcontaining bodies (14 Fig 7) It must also be kept in mind that asbestos may be of different types and the carcinogenic effects of these may differ Exposure to asbestos does not always lead to a permanent deposition of asbestos bodies in the lung and there is no simple relation between the degree of exposure to asbestos and the frequency of asbestos bodies in pulmonary tissue Thus *Vigliani* (23) found no asbestos bodies in the lungs in 10 cases of mesothelioma with a known

clinical history of exposure to asbestos A study from Cape Town (18) comparing asbestos mine workers in the North Western Cape and the Transvaal showed a similar frequency of asbestos bodies in lung sections from the two groups whereas the frequency of asbestos bodies in the sputa of living patients differed (35 per cent in males from the North Western Cape 10 per cent in males from the Transvaal)

SUMMARY

Cases of mesothelioma (27 of the pleura 8 of the peritoneum) in a ten year autopsy material (12 763 autopsies) are presented Thirty four have been re examined for the presence of asbestos bodies in the lungs The control series was composed of randomly chosen autopsy cases (34) of matching age and sex Asbestos bodies were found in 18 cases in the mesothelioma group and in 12 cases in the control series the differences is thus rather small In this regard the present study differs from most similar investigations *which report an evident difference* between the presence of asbestos bodies in cases of mesothelioma and in the control series We should like to stress the fact that a small number of asbestos bodies normally can be seen in lungs If a positive correlation between the presence of asbestos bodies and mesothelioma is to be *proven* bodies *must be numerous in the cases of mesothelioma* In the present investigation abundant bodies were found in 6 cases in the mesothelioma group and in 1 case in the control series

Add This material has earlier been presented to the Swedish Society of Pathology in Stockholm December 1966 by *Hagerstrand Odlund* At that *time the figures were* asbestos bodies were observed in 7 cases in *the mesothelioma group in 2 cases in the control group Only* classic bodies about the length of 70 microns had been counted We are very pleased because Doctor Meurman kindly participated in this work and re examined the material that it might be comparable with his own earlier work (14)

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THE THYMUS OF THE ADULT STRESSED DOG, A PREDOMINANTLY EPITHELIAL ORGAN

A Histological, Histochemical and Statistical Study

By

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Watney (1882) (35) was the first to describe epithelial cysts in the thymus of the dog *Hammar* (1905) (18) claimed that they occurred fairly frequently in this animal and (1921) (19) stressed the epithelial character of the thymus *Marne* (1928) (22) observed that they were more frequent in the endemic goiter region than elsewhere *Bargmann* (1934) (3) gives an account of them in the dog and claims that their amount increases with age so as even to dominate the histological picture of the thymus

There have been accounts of epithelial formations rosettes or cysts in the thymus of other animals cats pigs cattle rabbits mice and rats (*Hammar* 1905 (19) *Bell* 1906 (4) *Erdheim* 1906 (14) *Dearth* 1928 (9) *Tschassownikow* 1929 (34) *Selye* 1936 (28) *Ross et al* 1941 (27) *Plagge* 1944 1946 (25 26) *Davidson* 1951 (8) *Dunn* 1954 (12) *Arnesen et al* 1958 1961 (1 2) *Diderholm et al* 1958 (10) *Csaba et al* 1963 (7) *Cowan et al* 1963 (6) *Limhartova* 1964 (21) *Friedman et al* 1964 (15) *Oksanen* 1966 (23) *van Haelst* 1967 (17))

Dubois (1850) (11) regarded thymus cysts as pathognomic for syphilis in man, and in the extensive autopsy material of *Weise* (1939) (36) in the thymus epithelial formations which he termed Primitiv körperchen were generally distributed

The histochemical aspect of thymic cysts has been investigated in mice by *Smith* (1964) (29) and in rats by *Oksanen* (1966) (23) The epithelial cells of the cysts showed activity of non specific esterase succinic dehydrogenase acid and alkaline phosphatase and DPN diaphorase (NADH lipoamide dehydrogenase)

To the knowledge of the author there have been four histological accounts of cysts of the canine thymus but no study of their histochemistry and none of their incidence in different age groups in the respective sexes or in various conditions of disease

For this reason the present inquiry was made concerning the frequency of incidence of thymic cysts in the dog and their proportional relationship to the lymphocytes as well as the distribution of the two types of tissue in different age groups in the sexes and in association with various diseases At the same time a histochemical study was made of the localisation and activity of six different enzymes in the cells of these cysts The chemical reaction of the cysts was also examined by means of PAS staining and Alcian blue technique

MATERIAL AND METHOD

Random samples for this study were collected from autopsy material in the Department of Pathology of the College of Veterinary Medicine over the period 14 9 1965–1 4 1966 the total being 107 thymus specimens from dogs above 6 months of age The animals varied in age were of either sex and had died or had been destroyed in various conditions of disease The histological samples were taken from the precardial mediastinum on the ventral side of the oesophagus and the trachea For the most part the thymus was macroscopically recognisable only in young animals or in those which had died suddenly In emaciated individuals the mediastinum was sometimes a thin almost transparent membrane with roundish grey violet nodes of tissue 2–3 mm in diameter In fat dogs there was in the region of the precardial mediastinum a layer of fat up to 5 cm thick divided into sections reminiscent of the lobules of the thymus Marked hyperaemia was frequently observed in the precardial mediastinum

The samples were fixed in 10 per cent formalin embedded in paraffin sectioned at 4 μ and stained with haematoxylin eosine For the histochemical study thymic samples were taken from 8 dogs which had just died or been destroyed They were frozen with solid CO and sliced to a thickness of 1? μ in the cryostat microtome at —18 C or where the preparation was very fatty at —24 C. For the *acid and alkaline phosphatase* tests the cryostat cuts were fixed in ice-cold acetone and treated by *the Gomori method for a period of 30 minutes and 1 hour s incubation* respectively For the following reactions unfixed cryostat samples were used to ascertain *non specific esterase* by the *acetate method* for 3 minutes and for *succinic dehydrogenase* by the *nitro BT method* according to Nachlas incubation time being 20 minutes The specimens were furthermore examined for *DPN diaphorase (NADH)* by means of the *Nachlas Walker* and *Seligman* method applied for 30 minutes and *LAP (leucine aminopeptidase)* by the same method with an incubation time of 2 hours The PAS staining was carried out as recommended by *Hochiss* and *Steedman* The staining methods used are described in the manual of *Pearse* (1961) (24) Controls lacking the specific reaction agent were provided for each method

In order to try to obtain a comprehensive picture of the different elements in the histological preparations these were classified on a scale of 0–10 If no cysts were observed in the histological preparation the frequency was assessed at 0 while the highest incidence was signified by 10 in which case the preparation was more or less completely dominated by cysts Correspondingly the complete absence of lymphocytes was signified by 0 and the most abundant presence by 10 where the lymphocyte count was carried out in the thymus of a young healthy dog

RESULTS

Histological Study

In the histological examination 83 of the 107 canine thymus glands contained epithelial cysts There were considerable variations in the histological picture for example in the amount of lymphocytes hyperaemia and cysts *in their form and content* as well as *in the texture of*



Figs 1–2

Fig 1 Canine thymus Three epithelial cysts surrounded by lymphocytes and blood vessels In the left side adipose tissue following involution He × 40

Fig 2 Canine thymus An elongated epithelial cyst of indefinite form surrounded by adipose tissue He × 60



Figs 3–4

Fig 3 Canine thymus A part of an epithelial [illegible]st H e × [illegible]0
Fig 4 Detail of Fig 3 H e × 67[illegible]

the wall membrane The thymus of the healthy young individual contains a particularly large number of lymphocytes The lobules are clearly divided into cortex and medulla Hassal corpuscles are seldom observed in the adult dog although they are occasionally found in young individuals

In those thymus glands which diverged most from the above picture varying amounts of fatty tissue were observed which were divided into units corresponding to the thymic lobules The centre of these contained cysts of varying size often surrounded by lymphocytes (Figs 1 4) The forms of the cysts ranged from circular to narrow and oblong They were bordered by cube shaped or cylindrical ciliated epithelial cells The large sometimes oblong nucleus of the cells was situated closer to the basal membrane than to the ciliated end (Fig 5) The cysts frequently contained an eosinophilic PAS and Alcian blue positive structureless mass cell detritus degenerated cells and often some presumably post mortally desquamated intact ciliated epithelial cells Bloodfilled arteries and veins were directly connected with the cysts In many cases the wall of the cyst surrounded the blood vessel in such a way that the form of the cyst was distorted The cyst resembled a lake with a broken shoreline in which the blood vessels were like headlands or at some levels of sectioning like islands there being nevertheless always an epithelial layer of the cyst wall between the bloodvessels and the content of the cyst

In the most radically changed thymus glands with marked hyperaemia and a cyst epithelium of high intact and active appearance an abundance of enzyme activity in the epithelial cells could be shown histochemically Marked signs of *DPN diaphorase (NADH)* (Figs 11 12) *non specific esterase* (8) and *succinic dehydrogenase* were observed in the cytoplasm of these cilia epithelial cells The cilia were only faintly visible A small grained reaction of *alkaline phosphatase* was obtained in the cytoplasm in the nucleus a faint reaction in places and in the cilia a particularly marked reaction (Fig 9–10) *Acid phosphatase* caused a marked reaction in nuclei and cytoplasm (Figs 6–7) All cytoplasm reactions were stronger in the area between the nucleus and the cilia than between the nucleus and the basal membrane No traces of *LAP* activity were observed The content of the cysts was *PAS positive* and showed a light blue reaction to *Alcian blue*

However sometimes the picture of the thymus cysts show a different histological structure In some cases it was possible to see cysts of the form described But they appeared to be empty and their walls were covered with flat cells In these cases the thymus region showed no hyperaemia Many different variations between the extreme forms of cysts described were observed in the material



Figs 5–7

Fig 5 Ciliated epithelial cells on the wall of a cyst in the canine thymus H e × 1600

Fig 6 A part of an epithelial cyst in the canine thymus Acid phosphatase × 700

Fig 7 Desquamated epithelial cells in a canine thymus cyst Acid phosphatase × 1600



Figs 8–11

Fig 8 A part of an epithelial cyst in the canine thymus Non-specific esterase × 1100

Fig 9 A part of an epithelial cyst in the canine thymus Alkaline phosphatase × 760

Fig 10 Desquamated epithelial cells in the cyst of the canine thymus Enzyme activity is strongest in the cilia Alkaline phosphatase × 1500

Fig 11 A part of an epithelial cyst in the canine thymus DPN diaphorase (NADH) × 460



Fig 12

A granular enzyme activity in the cytoplasm DPN diaphorase × 1600

Presence of Cysts and Lymphocytes and their Relative Proportion

The material used in this study comprised 107 canine thymus glands In 83 cases or 77 5 per cent of the total cysts were found to be present On the basis of the mode of assessment mentioned in the section on Material and Method the average frequency of incidence of cysts was 4 15 and that of lymphocytes 2 80

A correlation analysis of the frequencies thus ascertained for cysts and lymphocytes revealed a highly significant negative correlation R was —0 383 The regression coefficient was B = —0 306

The Effect of Age

The age of the animals studied was known in 83 cases A correlation analysis gave the following results There was an almost significant positive relationship between age and the presence of cysts R = 0 283 B = 0 309

Between age and lymphocyte density again there was a highly significant negative correlation R = —0 535 and B = —0 773

The Influence of Sex

The incidence of cysts averaged 4 3 in the female and 4 6 in the male

The Role Played by Various Conditions of Disease in the Incidence of Cysts

The material was divided into groups according to different patho

logical conditions. Because of the small number of cases in the different groups the material could not be treated statistically. Table 1 shows those groups of different pathological conditions which diverged markedly from the average as well as the group called "varia" embracing all completely isolated cases and finally the groups which came closest to the average. The group designated "negative" comprises 8 animals which did not show any pathological anatomical changes and which were destroyed on account of maintenance difficulties as well as two individuals which had died immediately subsequent to trauma.

TABLE 1

Diagnosis	No. of cases	Average incidence of cysts	Average incidence of lymphocytes
Neoplasms	20	5.04	2.20
Diabetes and adipositas	7	6.14	0.86
Nephritis interstitialis *chronica (without uraemia)*	5	8.00	0.40
Other chronic inflammations	26	4.65	2.04
Varia	39	3.73	2.57
Negative	10	0.20	5.50
Total material	107	4.15	2.80

DISCUSSION

Histological examinations were carried out on a total of 107 canine thymus glands. Even in cases where it was not possible to recognise thymus tissue macroscopically, samples for histological study were taken from the precardial mediastinum on the ventral side of the oesophagus and the trachea. Only individuals over 0.5 years of age were included. Thymic cysts were ascertained in 77.5 per cent of the material. This figure is decidedly larger than the 20 per cent given by *Marne* (1928) (22). The reason for this may be that in the present work samples were also taken when the thymus could not be recognised macroscopically.

Between cyst and lymphocyte density there was a highly significant negative correlation (R = —0.383, B = —0.306). In other words an abundance of cysts occurred in connection with lymphocyte decrease, *i.e.* involution. Age and lymphocytes were found to be correlated negatively to a highly significant degree (R = —0.535, B = —0.773). *Hammar* (1905) (18) called this phenomenon age involution. Between cysts and age on the other hand there was only an almost significant positive relation (R = 0.283, B = 0.309). The incidence of cysts was thus only partly dependent upon the age of the

individual or upon age involution and in fact they were found to be associated above all with involution not deriving from age the accidental involution *Hammar* (1905) (18) observed in various cases of stress such as disease or hunger

The material was too small and heterogeneous to allow for statistical treatment of different groups of disease It was however possible to ascertain in some groups of disease average frequencies of incidence for cysts which did diverge from the rest While the average for the whole material was 4 15 in negative autopsies (10 cases) the average incidence of cysts was 0 20 a figure considerably lower than any other in the material This supports the findings of the correlation analyses of age cysts and lymphocytes The emergence of cysts is largely dependent upon external conditions of disease In a number of the disease groups the frequency of incidence of cysts showed an average higher than that of the total material Chronic interstitial nephritis (5 cases) averaged 8 00 diabetes and adipositas (7 cases) 6 14 neoplasms (20 cases) 5 04 and chronic inflammations (26 cases) 4 65 From this it may be concluded that thymus cysts occur abundantly in association with a variety of pathologic conditions They are not bound to any particular disease but are rather a sign of a general reaction of the organism to stress

Hammar (1905) (18) observed in the canine thymus the presence of cysts whose characteristics corresponded to those noted by the present author *Hammar* (18) however interpreted them as degenerative changes sequesteria whereas in the present work the ciliated epithelial cells surrounding the cysts were found to be intact and have the appearance of life and activity They were observed both in animals which had died from natural causes and in dogs which had been destroyed at various stages of disease Thus the abundant enzyme activity observed in the cells may be attributed rather to lively cell function than to degenerative processes The findings of the present study would seem to support more the opinion of *Weise* (1939) (36) *Arnesen* (1958) (1) *Clark* (1966) (5) and *van Haelst* (1967) (17) i e that these cells have a special function an endocrine significance

Variations in the proportional relationship between the thymus lymphocytes and the epithelial reticular cells associated with involution have often been noted *Greenberg et al* (1965) (16) in their histochemical and biochemical studies conclude that even the number of epithelial cells increases in various stress situations *Khussar* (1964) (20) again noted mitose activity occurring in the epithelial reticulum cells subsequent to X ray treatment and associated with lymphocyte loss The highly significant negative correlation noted in the present material between lymphocytes and epithelial cysts may also be interpreted as a sign of antagonism between the different types of cells Lymphocytes dominate in the young healthy animal while epithelial formations emerge in connection with disease

Szent Gyorgyi et al (1962) (30) using a biochemical technique isolated in the thymus two *active and antagonistic components* the growth promoting promine and the growth retarding retine In the opinion of these writers both are present in balanced proportion in the thymus of the healthy animal and thus cancel each other's influence *in thymus extracts*

Tallberg's studies (1966) (31 32) likewise indicate two possible influences in the thymus He found from the thymus of rat two thymus specific antigens

If in fact, as the above mentioned studies would suggest the thymus does contain two simultaneously present and mutually antagonistic tissues emerging in different proportions under different external conditions this might explain the fact that in innumerable thymus extract studies described in the literature (refer to *Tesseraux* 1959 (33)) conflicting results have been obtained especially in cases where the relationship of the thymus to various endocrine organs is investigated These studies is usually lacking on the histological picture of the thymus

SUMMARY

A histological study was carried out on 107 canine thymus glands macroscopically visible or already involuted taken from autopsy material of over 6 months of age but otherwise random sampled The specimens were taken from the precardial mediastinum In 77 5 per cent of the material cysts of varying size and form were observed The frequency of incidence of cysts varied The walls of these cysts were generally surrounded by cubic or cylindrical ciliated epithelium Between the frequencies of incidence of cysts and lymphocytes a highly significant negative correlation was ascertained likewise between age and lymphocytes while between age and cysts only in a most significant positive relation was found In young healthy dogs lymphocytes dominated the histological picture whereas the cysts dominated in chronically diseased cases The epithelial cells revealed the activity of alkaline and acid phosphatase succinic dehydrogenase DPN diaphorase (NADH diaphorase) and non specific esterase

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ON THE CHEMICAL PATHOLOGY OF INTERSTITIAL FLUID

2 *Intracellular and Interstitial Enzyme Activity in Experimental Granulomas*

By

Mikko Niemi[1] and Bengt Sylvén

Received 1 xii 67

The mononuclear phagocytes play a key role in chronic inflammations and in many non bacterial inflammatory processes Increased attention has therefore been focused on the morphological and cytochemical mode of reactivity of the inflammatory phagocytes in specific (*Grogg & Pearse* 1952) and non specific chronic inflammations (*Gedigk & Fischer* 1960) as well as in delayed hypersensitivity reactions (*Waksman* 1962) and in homograft rejection (*Perez Tamayo & Kretschmer* 1965)

Resting macrophages are known to show inconspicuous enzymatic reactions (*Grogg & Pearse* 1952 *Gedigl & Bontke* 1957 *Suter & Hulliger* 1960 a o) Morphological studies under the light microscope (*Metchnikoff* 1892) electron microscope studies (*Dumont & Sheldon* 1965) and cytochemical investigations (*Gedigk & Fischer* 1960) have shown increased activity in mononuclear macrophages in response to inflammatory stimuli It is known that active macrophages show increased activity of histochemically available lysosomal enzymes at the site of an inflammatory process (*Gedigk & Bontke* 1957 *Mustakallio & Niemi* 1966) Direct evidence of the intracellular events in the macrophages during the inflammatory process stem from the *in vitro* studies of *Cohn & Wiener* (1963) (reviewed by *Cohn* (1965)) who have shown that the macrophage responds biochemically to the act of phagocytosis by a rearrangement of its lysosomes which is subsequently followed by a discharge of the lysosomal enzymes to the phagocytic vacuoles and leakage of some of these enzymes to the extracellular space

The biochemical events taking place in macrophages *in vivo* are

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TABLF
Methods of

Assay	Dilution of orig sample	Puffer	Substrate
AcPhase	1 + 3	Citrate 0 05 M	p nitr phenyl phosphate
β glucoronidase	1 + 3	Acetate 0 1 M	Phenolphthalein β glucuronide
Aryl sulphatase	1 + 3	Acetate 0 2 M	p nitrocathechol sulphate
LNase	1 + 19	Pho phate 0 2 M	Leucyl β naphthyl amide
Total catheptic activity	1 + 9	Acetate 0 2 M	Urea denat bovine hemoglobin r edestine
Dipeptidase	1 + 9	Tris HCl 0 05 M	Alanyl l glycine
LDHase	1 + 9	Tris EDTA	Na pyruvate
GSSG reductase	no dilut.	Phosphate	Glutathione ox

largely unknown and little is known about the composition of the extracellular fluid environment of the reactive tissue It was therefore of interest to study the enzymatic pattern of the interstitial fluid of early granulomas in which the macrophages form the predominant cell type Further comparison with similar enzymatic data from solid tumours (cp *Sylven & Bois Svensson* 1964 1965) will appear in a subsequent paper

The present contribution will cover three particular aspects relating only to experimental granulomas namely

1) The intra and extracellular histochemical enzyme reactions
2) The occurrence of cell injury and lysis on the part of macrophages and
3) The enzymatic activity pattern of the cell free interstitial granuloma fluid

MATERIALS AND METHODS

Foreign body granulomas were produced in adult rats of both sexes by injecting aseptic suspensions of talcum powder preheated to about 140 C for 4 48 hr *From 2 to 5 ml of saline suspensions* were injected subcut or im int each r t n both sides of their back musculature Rats were killed at 4 7 11 28 and 64 days after the injection and pieces of the granulomatous tissue were proces ed f r histology and histochemistry

The nature of the inflammatory cell response wa assessed on stained smears of

I
Assay

Substrate conc	Activator added final conc	Final pH of react mixture	Incub temp	Incub time	References
0 0055 M	None	4 8	37	30 min	*Andersch & S c ypinski 1947*
0 0015 M	None	4 6	37	30 min	*Fishman & Bernfeld 1955*
0 0? M	None	5 5	37	1 h	*Roy 1958*
0 014 M	None	6 8	40	1 h	*Goldbarg & Rutenburg 1958* cp *Sylven & Bois 1967*
2 %	Cysteine 0 004 M	4 1	40	90 min	*Anson s* method as modified by *Ottosen & Sylven 1960*
0 ? M	Mg^{++} 0 01 M	7 8	40	1 or 2 h	*Linderström Lang 1929* cp *Sylven & Malmgren 1957*
—	None	7 ?	25°	0–3 min	Cp *Burgess & Sylven 1962*
—	None	7 6	25	0–15 min	*Racker 1955* cp microadapt *Malmgren & Sylven 1960*

central granuloma fluid and detritus taken directly for inspection In this way cases with bacterial contamination presenting a turbid fluid rich in leukocytes were grouped separately

Evaluation of cell injury—The frequency of irreversible injury in the cell population of non infectious granulomas was ascertained by the dye exclusion test of *Holmberg* (1961) Suspensions of macrophages and other cells were produced by shaking thin slices from the fibrous capsule and the central talcum detritus separately in saline at 37 C After centrifugation the free floating cells were resuspended in a 1 per cent solution of lissamine green and the frequency of cells permeable to the dye were counted Lysed cells and cell remnants were also noticed in sections for ordinary histology

Histological and histochemical techniques—Tissue pieces were either fresh frozen in iso pentane chilled in solid carbon dioxide or fixed overnight in formol calcium at +4 C The former pieces were cut in a cryostat maintained at −25 C Formol calcium fixed material was transferred to Holt s gum sucrose medium (*Holt* 1958) kept at +4 C for at least 24 hours and cut without any further treatment in the cryostat Alternate serial sections of each sample were taken for routine histological staining with haematoxylin and eosin (H & E)

Acid phosphatase (AcPhase) activity was demonstrated both in fresh frozen and formol-calcium fixed tissues by the techniques of *Gomori* (1952) and *Barka & Anderson* (1962) naphthol AS MX phosphate served as substrate in the latter technique and sodium β glycerophosphate in the former technique β Glucuronidase activity was visualized using naphthol AS BI β glucuronide as substrate and hexazotized pararosaniline as the coupler (*Hayashi* 19 4) Esterase activity was demonstrated at pH 6 8 by the technique of *Holt* (19 8) using 4 chloro 5 bromo indoxyl acetate as a substrate 10 M E600 (paranitrophenyl phosphate) was in some cases used as an inhibitor included both in a preincubation bath for 30 min and in the substrate mixture

For the demonstration of aminoacyl naphthylamidase activity L leucyl β naphthylamide (LNA) was used as a substrate The incubations were carried out at pH 5 5

6 and 6.5 for 2 hours in the medium of *Nachlas et al.* (1957) containing cyanide and added Mg^{++} ions. Only unfixed fresh frozen and dried sections were used for the demonstration of LNase activity.

In order to avoid artefacts control sections of all reactions were incubated in media without added substrate. *In the case of the LNase reaction* spurious dye crystallization was prevented by shortened incubation times and diminished dye concentrations. Another effective way to avoid crystallization was to add enzyme inhibitors such as 0.05 M EDTA or 0.005 M periodate.

Interstitial granuloma fluid was sampled at 4, 7 and 11 days but older granulomas were too dry for sampling. The granulomas were dissected from the deep side bluntly opened up by forceps and the free fluid was collected by glass capillaries with an *internal diameter of 0.1–0.3 mm without applying pressure to the tissue*. At least 20 to 200 µl of fluid could usually be obtained from each focus also in cases of "dry" granulomas; when necessary individual samples were pooled to obtain enough fluid for assay. Admixed cells were effectively removed in a capillary centrifuge.

Sampling of blood plasma and interstitial fluid from the peritoneal cavity (IP fluid) of normal rats of different age groups was performed as previously described (*Sylvén & Bois Svensson* 1965).

Biochemical assay techniques.—Fresh unfrozen cell- and blood-free interstitial granuloma fluid was suitably diluted and processed for the assay of protein content (*Naygar & Glick* 1954), degree of UV absorption and activity of a selection of enzymes. These included some of lysosomal origin and others, such as dipeptidases, considered to occur in a dissolved state in the cytoplasm. The total LNase activity was assayed with special interest although the precise subcellular compartmentalization still is an open question (cp. below). For comparison similar data were collected on fresh samples of normal rat plasma and IP fluid. All technical specifications have been compiled in Table 1. In the case of AcPhase, β-glucuronidase, aryl sulphatase, lactic dehydrogenase (LDH) and glutathione (GSSC) reductase standard assay methods were followed. In the case of LNase, acid proteinase and dipeptidase assays the pH distribution curves of activity were determined for each material separately in order to ascertain the optimum pH under present conditions of assay (Table 1). Considerations regarding enzyme stability *necessitate the use of fresh materials*: considerable losses of activity by storage over night at refrigerator temperature or by deep freezing and thawing have been noticed particularly in case of the metal-dependent dipeptidases, LNases and AcPhase as well as to a lesser degree with the cathepsins. The other 4 enzymes under study were less affected by freezing and could be processed the day after sampling.

Separate experiments are further recorded in the text in order to study the reasons for the low cathepic and dipeptidase activities of granuloma fluid. No separations have been made in order to characterize the composite nature of the different acid proteinases (mainly cathepsin B and D and possibly some E) responsible for the total catheptic activity assayed (cp. *Robert & Cambier* 1964).

RESULTS

Our interest was mainly attached to the first two weeks of early granuloma formation characterized by non-bacterial inflammatory chan

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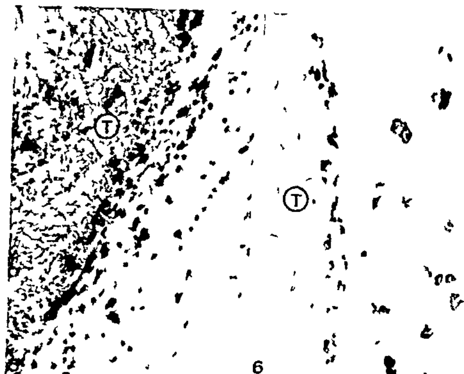
Figs 1–3

Fig. 1. A fresh frozen section of a 4 days old talcum (T) granuloma stained with H & E. A collection of mononuclear cells around the foreign body and signs of cell destruction. × 200.

Fig. 2. AcPhase activity in a 4 days old granuloma (Naphthol AS-MX phosphate as substrate). High enzyme activity around the talcum deposits and in p[illegible] as cular phagocytes. × 200.

Figs. 3 and 4. LNase reaction in 4 and 6 days old granulomata. Only the [illegible] live macrophages in the foreign body showed high enzyme activity. [illegible] 1.0 Fig. 4 × 200.


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Figs 5–6

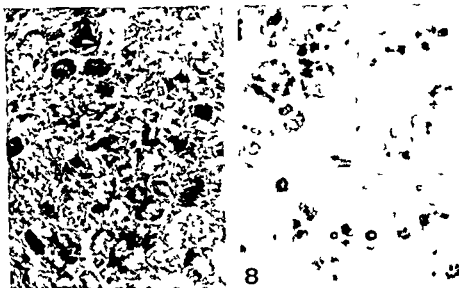
Fig 5 Non specific esterase activity in a formalin fixed section of a 4 days old granuloma Reactive macrophages showed distinct activity and some diffuse *extracellular reaction around the talcum (T)* as well × 300

Fig 6 The macrophages showing β glucuronidase activity were less in number but often contained large intracellular flags or were heavily stained throughout the cytoplasm × 300

ges and massive accumulation of active macrophages All cases subject to bacterial infection presenting a turbid fluid rich in leukocytes were discarded from histochemical evaluation but are for the purpose of the records mentioned under Section III

I *Histology and Histochemical Observations*

On the 4th day after injection the talcum deposits were surrounded by marked oedema and a cellular reaction containing a few leukocytes and masses of actively phagocyting macrophages forming a dense wall around the deposits (Fig 1) In 4 to 7 days old granulomas most of the enzymatic activity was concentrated to these cells The AcPhase (with both substrates) and ENase activities were especially prominent (Fig 2 3 and 4) The β glucuronidase and E600 resistant indoxyl esterase activities studied only in formalin fixed tissues were also very active in the most prominent macrophages in which large enzyme positive coalescent granules or vacuoles were seen (Fig 5 and 6) Macrophages located further outside the narrow reactive layer generally ex



Figs 7–8

Fig 7 All giant cells of a 28 days old granuloma showed intense LNase activity × 200

Fig 8 A smear from the central detritus of a 11 days old granuloma A number of large LNase positive bags in damaged macrophages × 600

hibited a strong AcPhase activity in many small granules but only a weak or inconspicuous LNase activity localized to a few small vacuoles per cell This tends to suggest that the two types of enzyme activity may not be located in the same subcellular compartment or may not *be activated under similar biological conditions*

The AcPhase and LNase reactions showed some diffuse cytoplasmic staining in macrophages as well as positive reactions also in the interstitial protein rich exudate (cp Section III) especially around the talcum particles The increased extracellular enzyme reactions suggest that enzymic proteins have leaked out into the interstitial phase

In granulomas older than 11 days the granulation tissue penetrated deeper into the talcum deposits massive phagocytosis by macrophages occurred and an increasing number of multinucleated giant cells was seen All the investigated histochemical activities were high throughout this granulation tissue including the fibroblast Especially marked activity was found in macrophages engulfing talcum particles as well as diffusely in the cytoplasm of giant cells (Fig 7) With a view to cells shed into the exudate cell injury and death was more prominent (cp below) Such damaged macrophages and other cells contained large enzymatically reactive vacuoles most cells were filled by one or a few giant LNase positive bags typical of advanced stag[illegible] cell injury and autolysis (Fig 8 *Sylvén & Lippi* 1965)

II *Frequency and Results of Cell Death*

Judging from the histological sections a moderate number of macrophages seemed to die in the course of talcum phagocytosis mostly about *the 7th to 11th days* of granuloma formation. Heavily vacuolized balloned cells were seen, nuclear fragmentation occurred and broken *cells were found*. That polymorphonuclear leukocytes eventually died was clear since they disappeared from the exudate *during the same* time. More exact information was however derived from the *lissamine green dye exclusion test* on suspensions of cells from different parts of the granulomas.

Briefly, about 1 per cent of cells suspended from the capsular layer were permeable to the dye at 4 and 7 days. This is possibly due to the fact that only a limited number of cells became detached. Suspensions of the central talcum layer generally contained after the 4th to 7th day up to 10 per cent of dead cells (Table 2). On the 11th day the clear central fluid contained few dead cells while the central detritus was estimated to contain about 60–100 per cent irreversibly damaged macrophages. Most of these cells were heavily loaded with talcum particles and may moreover have got a too long diffusion path for survival. The frequency of damaged and dead cells was much increased in infected granulomas (Table 2, Section B).

TABLE 2
Estimated Percentage of Irreversibly Damaged Cells in Different Parts of Talcum Granulomas

	Age of granulomas Days (number of cases)	Cell suspensions from the capsule %	the fluid %	the central [illegible]
A	4 (10)	~ 1	~ 1	1 10
	7 (11)	~ 1	1 – 10	1 10
	11 ([illegible])	11 – 50	1 – 10	80 100
B	11 (10)	[illegible]	60 90	70 100

A Non infected granulomas with clear fluid
B Infected cases with turbid fluid

Among the enzyme reactions the [illegible] again proved of value for the diagnosis of injured and autolysing cells. The appearance of very large vacuoles or "bags" with a strongly positive [illegible] reaction served as suggestive guidance (Fig 8).

III *Biochemical Observations*

The non infected cell and blood free granuloma fluid was water clear or slightly yellow it contained no detritus and did not clot spontaneously As compared with normal IP fluid the total protein content of 4 days old granuloma fluid was increased from about 3 to 5 8 per cent The protein content at 11 days was slightly lower about 5 2 per cent (ranges 4 4–6 1 per cent) No increased UV absorption was found at 260 mμ

The average figures of relative enzymatic activity per a volume basis presented a characteristic profile with a balance markedly in favour of certain lysosomal enzymes (Table 3) At 4 days the AcPhase β glucuronidase and aryl sulphatase activities were increased about 8–10 times over the normal levels of IP fluid The LNase activity showed an increase of about 10 times while the LDH and GSSG reductase activities were less increased The total catheptic activity was only slightly increased but the dipeptidase remained very low Later on at 7 and 11 days all activities went down except the cathepsins which showed a further rise During the sequence of these events no apparent correlations were noticed between enzyme activity and actual volume of granuloma fluid high enzyme activities were found both in very dry cases and in those with large amounts of fluid

The enzymatic pattern was on the other hand markedly changed in infected cases presenting a turbid leukocyte rich exudate containing a large percentage of dead cells (Table 3 bottom section) At 4 days all enzymatic activities including the cathepsins LDH and GSSG reductase were much higher only the dipeptidase activity remained low The subsequent variations with time were grossly related to the extent of cell death and release of lysosomal and other enzymes There was again a marked decrease of lysosomal enzyme activity with time with exception for the catheptic activity

It is fully realized that these *in vivo* activity data illustrate the situation in situ without due information of the possible influence by enzymatic inactivation with time presence of natural inhibitor levels rate of fluid transport and exchange etc A puzzling result was the low rise in total catheptic and negligible dipeptidase activity in clear fluid necessitating further experiments Granuloma fluid was thus added to partially purified cathepsin B and D preparations from liver The cathepsin B activity was markedly inhibited while the D activity remained uninfluenced (data to be published) The dipeptidase activity of liver homogenates was not influenced by added granuloma fluid for various lengths of time up to 4 hours Hence the granuloma fluid contained a cathepsin B inhibitor but not a cathepsin D or a dipeptidase inhibitor

The important fact remains that the cell free non infected granuloma fluid presented *a pattern of increased enzyme activity characterized by mostly lysosomal ones and LNases*

TABLE

Average Data on Protein Content and Enzymatic Activity of Cell Free

Material Age of granulomas and weight of rats		No of cases invest	Protein content %	AcPhase U/10 µl /30 min	β glucuronidase U/10 µl /30 min
Normal plasma					
Rats 60 g		5	5 5	1 64	[illegible]
Rats 300 g		14	6 2	1 60	6
Normal IP fluid					
Rats 60 g		5	[illegible]	0 16	8
Rats 300 g		14	3 0	0 16	15
A Clear granuloma fluid					
Rats 300 g	4 days	14	5 8	1 60	[illegible]
	7 days	27	5 6	1 36	66
	11 days	15	5 2	[illegible]	99
B Turbid granuloma fluid					
Rats 300 g	4 days	3	5 4	[illegible]	336
	7 days	2	5 5	3 60	416
	11 days	15	5 7	2 40	170

U = units Relative units cp *Burgess & Sylvén* (196? and 1963)

DISCUSSION

Tissue macrophages contain a substantial number of lysosomal hydrolases (review by *Woessner* 1965) *Their AcPhase activity is markedly* increased during phagocytosis at sites of inflammation and physiological or pathological cell disintegration (*Weber* 1963 *Woessner* 1965) The *in vitro* observations by *Cohn & Wiener* (1963) have demonstrated that this increase of enzymatic activity is most likely due to an intracellular rearrangement of the existing enzymes besides an apparent discharge of lysosomal enzymes to the phagocytic vacuoles some of the enzymes also leak from the macrophages to their surrounding medium The results presented in this paper fall well in line with these investigations Thus a very intense intracellular histochemical staining of reactive macrophages for some lysosomal enzymes was demonstrated both in the form of cytoplasmic granules and a *diffuse* over all staining of the cytoplasm On the other hand the LNase reaction *regularly produced one or a few heavily stained intracellular* vacuoles Moreover in the histochemical specimens the LNase reaction *product could often be seen extracellularly* Additional observations on the appearance of giant LNase positive bags indicate serious cell damage and cell death when previous experiments on injured cells and autolytic tissues are taken into account (*Sylven & Tippi* 1965) It was possible in the light microscope to identify autophagic vacuoles by their intense LNase reaction at about pH 6 This conclusion is strongly

nterstitial Granuloma Fluid (Cp Table 1 and Text)

Aryl sulphatase % hydrolysis/ 10 µl/hr	LNase % hydrolysis/ µl/hr	Cathepsin % hydrolysis/ 10 µl/hr	Dipeptidase µl NaOH/ µl fluid/hr	LDH U/10 µl/min	GSSC reduct U/10 µl/min
1 4	–	4 7	–	5	2
0 8	0 7	7 3	0 05	15	2
< 0 8	–	–	–	55	2
< 0 8	0 4	10 1	0 95	75	4
13 6	5 3	23 3	0 65	360	15
4 2	2 2	16 9	0 30	285	8
4 2	1 7	62 6	0 20	280	7
48 8	14 0	60 7	1 20	865	37
61 0	13 1	13 3	0 90	820	25
13 6	4 5	68 7	0 65	1375	42

supported by our results with counts of the non viable cells Furthermore applying the dye exclusion test and the histochemical reactions directly on cell smears from granulomas the presence of large intra cellular bags with a strongly increased LNase reaction could be definitely localized to injured cells It is also of interest that an increased LNase reaction constitutes an early sign of radiation damage in salivary gland and lymph node cells (*Greenspan et al* 1964) Thus both the dyeexclusion test and the LNase reaction appear useful for the diagnosis of injured cells

Little is known about life span of macrophages at the site of the inflammatory process (*Policard* 1957) Previous (*Allison* 1965) and present results illustrate how a non bacterial inflammatory reaction is associated during the first two weeks by some cell death on the part of macrophages floating free in the exudate

Turning to the biochemical data it seems conceivable that most of the exudate has accumulated by increased filtration from the blood the protein content of granuloma fluid was raised to a level close to that of plasma and was by far higher than the normal filtration rate of about 45 per cent of interstitial fluid Tentative correlations could equally well be made between granuloma fluid v normal plasma or interstitial IP fluid at a per volume basis When calculated per a protein basis it should however be noted that several enzymatic activities are higher in IP fluid than in the blood (Table 3 cp *Burgess & Sylvén* 1963 *Sylvén & Bois Svensson* 1965) due to add d enzymatic terms

from local cellular sources Furthermore there is little doubt that the major part of the locally increased enzymatic activities in the granuloma fluid is derived from leaking and/or damaged macrophages in conformity with the *in vitro* observations by *Cohn & Wiener* (1963) A release of lysosomal enzymes from injured cells has also been suggested by *Thomas & Weissmann* (1962) Additional very *considerable* enzymatic terms are further contributed as result of extensive cell death in infected cases of granulomas (Table 3 bottom part) For lack of detailed dynamic data further discussion of the relative magnitudes and time relations of these enzymatic activities have to be postponed

Finally the implications of such increased enzymatic activities of the extracellular fluid as to the course of an inflammatory process might be considered Referring to a delayed type of hypersensitivity reaction *Waksman* (1962) discussed the possibility that macrophages by release of enzymes might initiate an extracellular proteolysis of antigenic proteins *Curran & Clark* (1964) have further demonstrated that macrophages secrete materials into their external milieu which bring peritoneal implants of denaturated proteins into solution even at some distance from the cells (cp the extracellular proteolysis by tumor explants of *Santesson* (1935)) It remains to be seen whether the cathepsins of the granuloma fluid may have a similar effect The naphthylamidases are active at a physiological pH range (*Sylvén & Bois* 1962) they may have a bearing on the liberation of bradykinin in local inflammatory compartments (*Hopsu Havu et al* 1966) and may thus excert an important physiological function

SUMMARY

Experimental talcum granulomas in rats were investigated histochemically for the presence of some lysosomal hydrolases (acid phosphatase β glucuronidase and non specific esterase) and aminoacyl naphthyl amidase activities Cell free interstitial fluid was further collected from 4 to 11 days old granulomas for biochemical assay of lysosomal and other enzyme activities The macrophages of the reactive tissue showed intense histochemical enzyme activity Coarse intracellular vacuoles and a diffuse cytoplasmic reaction appeared during phagocytosis Leakage of enzymatic proteins into the extracellular space was likewise observed The giant cells and fibroblasts of the older granulomas were also enzymatically active

Microscopic evidence of cell death was obtained by ordinary histology and by means of dye exclusion tests on cells suspended from different parts of the granulomas Up to 100 per cent of irreversibly damaged macrophages were found in the central detritus while few dead cells were found in the capsular layer

The enzymatic composition of the cell free granuloma fluid showed a balance markedly in favour of lysosomal hydrolases and aminoacyl naphthylamidase activity This is interpreted as being caused primarily by enzyme leakage from active macrophages during phagocytosis

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RENAL BIOPSIES FROM PATIENTS WITH A HIGH ANALGESIC INTAKE

Histological and Bacteriological Study

By

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In the course of the past 14 years it has been repeatedly reported, especially from Switzerland and Scandinavia, that a high analgesic intake may entail chronic renal disease. *Spühler & Zollinger* (1953) were the first to call attention to this problem, pointing out that many patients with chronic interstitial nephritis had a history of a high intake of analgesics containing phenacetin. They interpreted this nephropathy as a toxic phenomenon, bacterial infection, if any, being of secondary importance.

Numerous clinical and experimental studies have been performed in an effort to throw light upon the problems concerning the nephrotoxic effect of analgesics. The results of these studies have frequently been inconclusive or directly conflicting, and in recent years this has given rise to animated discussions.

The reason why it is so difficult to decide whether the intake of analgesics in large quantities may involve toxic damage to the kidneys is perhaps that most clinical and pathological studies on this category of patients have been done at a time when the patients have had bacterial infection of the urinary tract. In that case the clinical as well as pathological appearances may be identical with the picture of chronic pyelonephritis. Consequently, it has been widely believed that in fact these patients develop chronic pyelonephritis.

In an effort to approach the solution of this problem, renal biopsies were obtained from a group of patients with a high analgesic intake, and the histological findings were compared with the bacteriological findings in the kidneys and urine.

MATERIAL AND METHOD

All patients admitted to Medical Department C of Bispebjerg Hospital, Copenhagen are carefully questioned about their intake of drugs. Owing to the particular interest displayed in the analgesic problem in this Department great stress is laid on accurate data concerning the analgesic consumption.

During the period August 1963 to August 1964 renal biopsies were obtained from 30 consecutive patients who had had a daily consumption of at least 1.5 g of phenacetin and/or acetylsalicylic acid through at least 10 years. These patients ranged in age from 27 to 71 years. 25 were females and only 5 males (Table 1). The majority had been admitted for headache or dyspepsia, only a very few with urinary trouble.

TABLE 1

Age Distribution and Sex Ratio of 30 Patients with a High Analgesic Intake

Age		21–30	31–40	41–50	>51
No. of patients	♀	2	6	7	10
	♂	0	1	1	3

The biopsy technique was that described by *Iversen & Brun* (1951). The specimen is a tissue cylinder 15–40 mm in length and 1.7 mm in diameter. Two biopsies were removed from each patient. One was used for histological and the other one for bacteriological study. The biopsy to be used for the histology was immediately fixed in Lillie's fixative and carried after 1–2 hours through the usual processes, embedded in paraffin and cut into serial sections. The staining methods were haematoxylin eosin, van Gieson Hansen and periodic acid Schiff (PAS). The preparations were examined in an ordinary light microscope.

The tissue specimen to be studied bacteriologically was placed in a sterile serum broth from which it was removed as soon as possible, at the latest after two hours, and crushed in a sterile mortar. The crushed tissue was then suspended in a new portion of serum broth and seeded on blood agar plates, on modified Conradi Drigalski plates, and on a thioglucollate medium to detect anaerobic bacteria if any. The plates were incubated at 35° C and read at the end of 24 and 48 hours. Thereafter the colonies were counted and the bacteria isolated and identified. The serum broth in which the renal tissue had been placed primarily was also incubated at 35° C to ascertain whether bacteria had been present on the surface of the biopsy. There might have been a question of contamination during the removal of the biopsy or of bacteria from the renal tissue proper. It should be emphasized that the patients had not received antibiotic treatment prior to the investigation.

On the last two days before and on the day of the biopsy procedure cultures of the urine were done. These cultures were made on mid stream urine (*Ffersoe et al.* 1963) diluted with sterile saline partly 1/20 and partly 1/200. 0.2 ml of these dilutions were seeded on blood agar plates, on blood agar plates pretreated with alcohol to prevent swarming of Proteus, and on modified Conradi Drigalski plates which were then incubated at 35° C and read at the end of 24 and 48 hours. The colonies were counted and representative colonies, if any, were removed for identification. A bacterial count of 10^5/ml urine or more was taken to indicate infection. No anaerobic cultures were made from the urine.

The identification of the cultured bacteria was done by the methods used at Statens Seruminstitut, Copenhagen. A few renal function studies were done, *inter alia* determination of the serum creatinine concentration, creatinine clearance, and the Addis Shevky concentration test.

RESULTS

Table 2 lists the patients' data. Half the patients were women under 50 years of age (Table 1).

It was endeavoured to obtain a tablet history as accurate as possible The patients often feel ashamed of their high drug intake and therefore pretend that they have taken and are taking less than they are The daily intake was usually between 6 and 12 tablets in a few cases up to 20 or even 50 tablets This intake had been going on for 10–50 years None of the present patients had been taking powders only tablets The most used analgesics are Codyl® Codeiphen® Celonid® Saridon® Coffizin® Coffiplex® and Aspirin® A number of these drugs contain per tablet 250 mg of phenacetin as well as of acetyl salicylic acid On the basis of the history the total intake of phenacetin and acetylsalicylic acid is estimated to have ranged from 10 to 50 kg of each of the named substances In other words there is a question of actual abuse An estimated total intake between 10 and 20 kg of each of the two substances was most common There is no demonstrable relationship between the size of the total dose and the renal function assessed by the creatinine clearance and the Addis Sheyky concentration test

The histological appearances of the renal tissue were fairly uniform Most tissue specimens contained cortical as well as medullary tissue in a few cases also papillary tissue Only one biopsy contained exclusively cortical tissue (No 972)

The glomeruli were normal in the great majority of cases However most specimens showed a few hyalinized glomeruli and biopsies from five patients (Nos 949 960 975 978 and 674) contained a number of hyalinized glomeruli In a few cases periglomerular fibrosis was observed

The distal part of the nephron comprising the loop of Henle a few distal tubules and collecting tubules showed degenerative changes The epithelium was in many sites atrophic or desquamated Frequently there would be thickening of the basement membrane A few tubules were dilated others atrophic (Figs 1 2 4) Table 2 includes a semi quantitative evaluation of the tubular changes which were in some cases mild (+) in others moderate (++) and in others again severe (+++) The proximal tubules were nearly always normal

In all the biopsies there was connective tissue proliferation localized to the medullary and papillary tissue while no or only very slight connective tissue proliferation was seen in the cortex It was a characteristic finding that the amount of connective tissue increased from the outer medullary zone towards the papilla which in some cases showed fibrous degeneration and obliterated structure (Figs 5 6) Table 2 gives the semiquantitative estimation of the connective tissue proliferation + signifying mild ++ moderate and +++ considerable connective tissue proliferation

In seven biopsies (Nos 947 975 976 986 997 1021 and 1028) the papillary and medullary tissue had undergone complete fibrous degeneration the ordinary structure was obliterated and there were areas of

TABLE

Survey of Various Data for 30 Patients with a High Analgesic Intake

Biopsy no	Sex	Age	Daily dose no of tablets	Duration of consumption year	Total dose kg phen	Total dose kg acet	Drugs
946	♀	50	6–10	20	14	14	codyl
947	♀	65	6–10	30	16	16	codyl coffazin magnyl
949	♀	60	6–10	20	29		phenacetin
952	♂	34	10–20	20	29	29	codyl coffazin
953	♀	27	8–12	10	10	10	codyl
959	♀	5[illegible]	6–10	20	11	11	codyl coffiplex
960	♂	61	6–10	45	34	34	codyl saridon
972	♀	31	6–10	12	10	10	codyl
975	♀	39	10–12	20	22	22	codyl coffazin
976	♀	45	10–12	20	22	22	codyl coffiplex
978	♀	56	6–12	40	30	30	codyl
986	♀	60	6–10	30	22	22	codyl
997	♀	65	6–10	45	32	32	codyl magnyl
999	♀	48	6–10	25	18	18	codyl magnyl
1001	♀	27	6–12	13	12	12	gelonida saridon
1003	♀	39	15–18	25	30	30	codyl coffazin
1006	♀	37	6–8	20	15	15	codyl magnyl
1009	♀	45	8–10	20	18	20	codyl magnyl
1021	♂	50	6–10	30	22	22	saridon coffazin
1028	♀	3[illegible]	6–10	18	13	13	codyl coffazin
1037	♀	49	6–10	12	10	12	codyl magnyl
674	♂	67	10–12	40	36	36	codyl coffiplex
1042	♂	55	6	30	16	16	codyl
1044	♀	38	10–16	12	17	17	codyl coffazin
1046	♀	71	2 3	50	10	10	codyl
1056	♀	50	6–8	30	16	16	saridon
1066	♀	45	16–20	25	45	45	codyl coffazin
1074	♀	5[illegible]	10–16	20	20	20	codyl
1137	♀	67	6–8	30	16	16	codyl
1145	♀	66	10–12	20	18	18	codyl

necrosis (Fig 3) None of these biopsies showed an infiltrative zone of leucocytes around such necrotic areas

Eight biopsies exhibited moderate (++) to considerable (+++) infiltration with inflammatory cells i e lymphocytes and plasma cells and a few granulocytes In the remaining 22 biopsies there was very little (+) or no inflammatory infiltration (Table 2)

The vessels were *normal in all the biopsies*

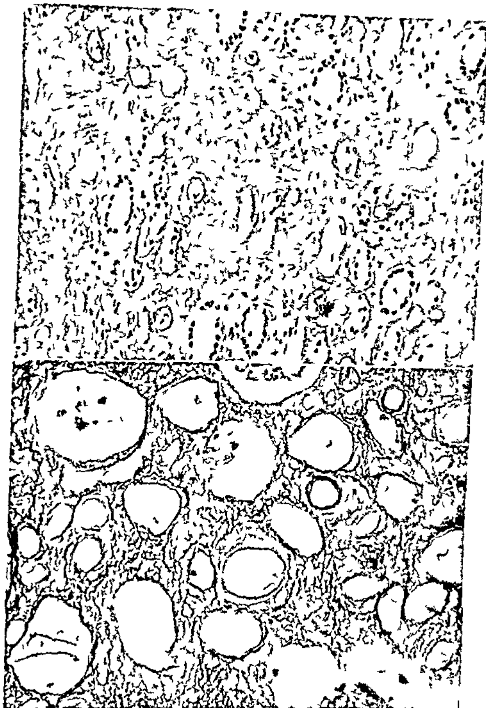
In Table 3 the biopsy material is divided into 2 groups Group 1 comprises patients in whom no interstitial inflammatory infiltration was demonstrable in the renal tissue Group 2 patients with such interstitial inflammatory infiltration (cf Table 2) This is then plotted against the various *parameters* It is apparent that the estimated consumption of analgesics is equally great in both groups

2
In Addition the Semiquantitative Evaluation of the Histological Renal Changes

Creatinine clearance ml/min	A ldis Sheck	Urinary tract infection	Voided papilla	Bacteriological finding in urine	Bacteriological finding in renal tissue	Tubular degeneration	Fibrosis	Interstitial infiltration
30	1016	—	—	10^5	—	+	++	—
5	1010	—	—	—	—	++	+++	—
18	1011	+	+	—	—	++	+	+++
50	1014	—	—	—	—	++	++	—
55	1011	—	—	—	—	+++	+++	+
25	1014	+	+	—	—	++	++	+
55	1014	—	—	—	—	+	++	++
95	1024	—	+	—	—	—	—	—
50	1010	+	—	—	—	+++	+++	—
10	1014	+	—	10	—	+++	++	+++
20	1016	+	—	10	—	+++	+	+++
20	1012	+	—	10	—	+++	+++	+
30	1014	+	—	10	—	+++	+++	—
70	1018	—	—	—	—	+	+	—
70	10'6	—	—	—	—	+	+	—
60	1024	—	—	—	—	+	+	—
60	10 0	—	—	—	—	+	++	—
90	1022	—	—	—	—	+	++	—
40	1024	—	—	—	—	++	++	—
20	1014	+	—	—	—	++	++	++
30	1016	—	—	—	—	++	+	++
5	1011	—	+	—	—	+++	+++	+
70	1022	—	—	—	—	++	++	—
110	1018	—	—	—	—	++	++	—
70	1021	+	—	—	—	++	++	—
25	1014	—	—	—	—	++	++	+
80	1025	—	—	—	—	+	+	—
'0	1010	+	+	10^5	—	++	+	++
30	1024	—	—	—	—	++	++	—
45	1021	+	—	—	—	++	++	++

From the table it is evident that four of the patients of each group had had symptoms of recurrent urinary infections One and two pa tients respectively had had symptoms of urinary infection and had also voided papillary tissue Two patients of the group without interstitial inflammatory infiltration had voided papillary tissue but had never had symptoms of urinary infection The papillary tissue had been histologically confirmed Fifteen and two patients respectively h d never had any urinary symptoms and at the time of the exam on they were not aware of any renal disorder

The renal tissue was sterile in all the examined cases B ia were cultured from the urine of only three patients of each g p These bacteria were *E coli* in a quantity of 10 bacteria/ml urine In all the others the urine was sterile on repeated culture



1

Figs 1–2

Fig 1 Tubular changes in the medulla The epithelium is atrophic or le Some tubules are dilated others atrophic There is considerable tissue proliferation (Biopsy no 9/5)

Fig 2 The same changes as in Fig 1 but the tubular chanc more There is thickening of the basement membrane (Bio 956)



Figs 3–4

Fig 3 Fibrou i eneration and necrosis of the papilla (Biopsy no 947)
Fig 4 Modera t bular degeneration From the cortico medullary junct on (B opsy n 9



Figs 5–6

Fig 5 Sclerotic degeneration of the papillar tissue (Biopsy no 997)
Fig 6 Fibrous degeneration in the medulla (Biopsy no 1071)

TABLE 3

The Material Grouped by the Occurrence of Interstitial Infiltration in the Renal Tissue This is Plotted against the Various Parameters

		Histological changes	
		0 Interstitial infiltration	+ Interstitial infiltration
No of patients		22	8
Abuse (kg)		20	21
Symptoms from the urinary tract	0	15	2
	infection	4	4
	inf + voided papilla	1	2
	voided papilla	2	0
Bacteriological findings in the renal tissue	0	22	8
	+	0	0
Bacteriological findings in the urine	0	19	5
	+	3	3
Creatinine clearance (ml/min)	= > 70	9	0
	20 – 69	11	2
	< 20	2	6
Addis Shevky	= > 1022	9	0
	1015 – 1021	4	3
	< 1014	9	5

In the group without interstitial inflammatory infiltration in the renal tissue 9 patients had a creatinine clearance of 70 ml/min or over and 7 patients could concentrate their urine to a specific gravity of 1022 or over All patients in the group interstitial inflammatory infiltration of the renal tissue had impaired renal function As already mentioned the majority of patients in this group had a history of urinary symptoms When also considering the result of the renal function studies this might indicate that the renal disease was more advanced in patients where histological examination of the renal tissue showed interstitial inflammatory infiltration than in those without this histological abnormality

The case histories of the 30 patients are fairly uniform The majority had been taking the tablets for headache Four characteristic histories will be given below

Case 1

A 34 year old single man with a family history of migraine Since puberty he had been taking 10–20 mixed analgesic tablets daily Never any urinary [illegible] During a stay in the Neurological Department his serum creatinine concentration [illegible] was found to be elevated The total intake of phenacetin and acetylsalicylic [illegible] is estimated to have been 29 kg of each

Haemoglobin concentration 11 0 g/100 ml E S R 22 mm sulphaemoglobin concentration 2 1 g/100 ml, methaemoglobin concentration 1 0 g/100 ml [illegible] creatinine concentration 1 6 mg/100 ml creatinine clearance 45 ml/min [illegible] Shevky concentration test 150 ml/1014 urinary pH 5 31 urinary protein [illegible] urine no growth B P 125/70 mm Hg [illegible] pyelography papillary [illegible] in the right

Histological examination of renal biopsy (No 9a2) [illegible] normal apart from very mild periglomerular fibrosis Proximal tubules [illegible] in the [illegible]

the tubular segments were slightly dilated with an atrophic degenerated epithelium. Quite some connective tissue proliferation. Vessels normal. No interstitial infiltration (Fig. 4).

Case 2

A 6[illegible] year old single woman who had ever since her youth been taking 6–10 analgesic tablets daily because of a hip damage. During the past 10 *years or so recurrent symptoms of urinary infection.* Papillary tissue had never been observed in the urine. Total intake of phenacetin and acetylsalicylic acid estimated as 32 kg of each.

*Haemoglobin c*oncentration 11.5 g/100 ml, E.S.R. 16 mm, sulphaemoglobin concentration 0, methaemoglobin concentration 0.8 g/100 ml, serum creatinine concentration 0.8 mg/100 ml, creatinine clearance 30 *ml/min,* Addis-Shevky concentration *test 75 ml/1011,* urinary pH 5.12, urinary protein 0, culture of the urine: 10⁵ *E. coli*/ml urine. B.P. 140/90 mm Hg. Iv. pyelography normal.

Histological examination of renal biopsy (No. 997): In the cortex 17 glomeruli, *10 of which were hyalinized;* the others were surrounded by very mild periglomerular fibrosis. Peritubular fibrosis around a few of the proximal *tubules* in the cortex. Medullary tissue characterized by pronounced connective tissue proliferation, *atrophy and degeneration of the* epithelium in the loop of Henle, distal tubules and collecting tubules. In places the lumen was slightly dilated, in other places atrophic. In this biopsy there was ample papillary tissue with sclerotic *degeneration, in places necrotic with obliterated structure.* No interstitial infiltration. Vessels normal (Fig. 5).

Case 3

A 50 year old man with diffuse headache through many years. For about 30 years he had been taking 6–10 analgesic tablets daily. The estimated total intake of *phenacetin and acetylsalicylic acid was 22 kg of each. Never urinary trouble.* The patient was admitted because of angina pectoris.

Haemoglobin concentration 11.5 g/100 ml, E.S.R. 4 mm, sulphaemoglobin concentration 0, methaemoglobin concentration 0, serum creatinine concentration 1.1 mg/100 ml, creatinine clearance 70 ml/min, Addis-Shevky concentration test 100 ml/1021, urinary pH 5.50, urinary protein 0, culture of the urine: no growth. B.P. 140/90 mm Hg. Iv. pyelography normal.

Histological examination of renal biopsy (No. 1021): Apart from mild periglomerular fibrosis the glomeruli were normal. In the medulla considerable connective tissue proliferation. Atrophy and degeneration of the epithelium in a number of Henle's loops. This biopsy contained papillary tissue with marked fibrous degeneration. Vessels normal. No interstitial infiltration (Fig. 6).

Case 4

A 38 year old woman who had been suffering from diffuse headache for many *years. Through 10–15 years a* daily intake of about 18 analgesic tablets. The estimated total intake of phenacetin and acetylsalicylic acid was *17 kg of each. The patient* had never had any urinary trouble. Admitted to the Neurological Department to have the cause of her headaches elucidated.

Haemoglobin concentration *12 g/100* ml, E.S.R. *2 mm,* *sulphaemoglobin concentration* 2.1 g/100 ml, methaemoglobin concentration 0.2 g/100 ml, serum creatinine *concentration 0.8 mg/100 ml,* creatinine clearance 110 ml/min, Addis-Shevky concentration test *40 ml/1018,* urinary pH 5.10, *urinary* protein 0, culture of urine: no growth. B.P. 115/75 mm Hg. Iv. pyelography normal.

Histological examination of renal biopsy (No. 1011): Apart from mild periglomerular fibrosis the glomeruli were normal. A few of the proximal tubules were surrounded by mild peritubular connective tissue proliferation. In the medulla pronounced connective tissue proliferation, atrophy and degeneration *of the epithelium of many Henle loops,* distal tubules and collecting tubules. Vessels normal. No interstitial infiltration.

DISCUSSION

Renal biopsies were obtained from 30 consecutive patients with a *high analgesic intake in order* to investigate whether a high analgesic

intake damages the kidneys and to assess the role of bacterial infection in the development of the nephropathy which so commonly affects these people

All the biopsy specimens exhibited histological changes though varied as is apparent from Table 2 One biopsy (No 972) contained only cortical tissue which was normal but the patient had repeatedly voided histologically confirmed necrotic papillary tissue It is reasonable to assume therefore that the medullary and papillary tissue would have shown changes similar to those found in the other biopsies

It is apparent from the present study that patients with a high analgesic intake may have well preserved renal function in spite of severe histological changes in the renal tissue The finding of a normal creatinine clearance in a number of the patients is not surprising in view of the slight glomerular changes The histological examination goes to show that the changes affect especially the medullary and papillary tissue It is strange therefore that the concentration ability is so relatively well preserved in some of these patients Thus the conventional clinical renal function tests cannot disclose with any certainty whether patients with analgesic abuse are suffering from renal disease *Sorensen*s (1960 1963) conclusion that a finding of a normal creatinine clearance and Addis Shevky concentration test in patients with a high analgesic intake should be taken to mean that analgesics do not damage the kidneys is therefore hardly correct To ascertain whether all persons with a high analgesic intake develop renal disease or whether individual factors are operating renal biopsies must be obtained from these patients to a far greater extent than hitherto

The renal tissue was sterile in all the present cases It was only in six cases that bacteria were cultured from the urine As already mentioned only one third of the patients had had symptoms or signs of urinary infection On the whole the result of bacteriological studies indicates that bacterial infection is not a decisive factor in the development of the renal disease in these patients

The histological changes were in all essentials identical with those described by *Gloor* (1961) in so called chronic sclerotic interstitial nephritis Gloor interpreted this nephropathy as a toxic phenomenon bacterial infection being merely of secondary importance *Rubinstein et al* (1964) studying renal biopsies from a few patient with high analgesic intake pointed out that the interstitial fibrosis was more pronounced and the interstitial infiltration less pronounced in these patients than in cases of pyelonephritis These findings are in keeping with the present observations Only eight of the present biopsies showed major interstitial infiltration and in these cases the histological appearances were identical with those seen in pyelonephritis Six of these patients had had clinical symptoms and signs of pyelonephritis The renal function was on the whole poorer in these patients than in

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THE "INVERSION STAGE" OF EXPERIMENTAL MOUSE AMYLOIDOSIS DETERMINED BY THE ACCELERATING EFFECT OF NITROGEN MUSTARD AFTER VARIOUS LENGTHS OF PRETREATMENT WITH CASEIN

By

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The first observations of the enhancing effect of nitrogen mustard on the development of experimental amyloidosis in mice was reported by *Teilum* in 1954 In his original experiment mice pretreated with daily injections of casein for 4 weeks were subjected to a subsequent treatment for one week with nitrogen mustard injected subcutaneously as 2 5–5 0 mg/Kg every second day The resulting change from a slight to a very heavy spleen amyloidosis in such treated animals was verified by comparison with spleen biopsies obtained immediately before the initiation of the accelerating nitrogen mustard injection series

This accelerating effect of nitrogen mustard on the formation of experimentally induced amyloid in mice has been repeatedly utilized in a number of experiments performed in this laboratory (*Werdelin & Ranlov* 1966 *Ranlov & Werdelin* 1967 *Ranlov* 1967b)

According to the two phase cellular theory of local amyloid secretion (*Teilum* 1964) it is to be anticipated that a compound which accelerates amyloid formation when applied during the second (amyloid) phase will prove an inhibitory effect on the development of amyloid when applied before the onset of and during the initial pyroninophilic (pre amyloid) phase This was shown to be true for cortisone the accelerating effect of which was demonstrated by *Teilum* (1952) while *Christensen* (1961) showed a markedly diminished incidence and severity of the resulting amyloidosis in mice treated with cortisone before and during the first weeks of casein treatment Similarly it has been shown that nitrogen mustard applied from the beginning of [illegible] treatment resulted in prevention or even inhibition of the subsequent amyloidosis in mice (*Ranlov* 1967a)

Thus during the course of an amyloid inducing treatment there

This investigation was supported by a grant from [illegible] of *Frederikssund Soren Alfred Andersens Legat*

seems to exist a critical moment *before* which acceleration of amyloid formation can not be achieved and *after* which such an acceleration for example, with nitrogen mustard may be achieved provided proper dosage This turning point may accordingly be considered to mark the so called Inversion Stage between the two well defined phases of experimental amyloid formation in the mouse

In order further to define the time of onset and the approximate duration of this Inversion Stage the following experiment was designed

MATERIAL AND METHODS

The material comprised 140 adult (3–4 months of age) C3H mice of an inbred strain 70 animals were females After randomization to avoid box effects they were divided into 7 groups with an equal sex distribution within the single group

Thus each group consisted of 10 male and 10 female mice Each animal in all groups received daily subcutaneous injections of a 5 per cent solution of casein in 0.25 per cent NaOH 6 times a week The first group received a total of 8 casein injections per animal the second group 10 the third group 12 the fourth group 14 the fifth group 16 the sixth group 18 and the seventh group received a total of 20 casein injections per mouse

On the day of the last casein injection each group was divided one half of the group (10 mice sex distribution equal) received subcutaneously ½ ml of a solution of nitrogen mustard in normal saline[1] the injected amount representing 0.05 mg of nitrogen mustard (Frasol®) each animal received a total of 3 injections with two days intervals and was killed the day after the last nitrogen mustard injection The other half of each group served as controls and were similarly injected with ½ ml of normal saline subcutaneously receiving a total of 3 injections every second day and accordingly being killed the day after the last saline injection

Spleen liver kidney adrenals and intestines were fixed in 10 per cent neutral buffered formalin for 24 hour After being embedded in paraffin sections were cut 5 microns thick and stained with haematoxylin eosin methyl green pyronine alkaline Congo red and with the P A S technique

Coded sections were examined independently by the two authors and the individual degrees of spleen amyloidosis were estimated by means of the semiquantitative technique described by Christensen & Hjort (1959)

RESULTS

As shown in Fig 1 it was impossible to obtain an acceleration of the casein induced amyloidosis earlier than after 12–14 casein injections After 12 days only one out of ten casein nitrogen mustard treated mice developed spleen amyloidosis and only to a very slight degree After 14 casein injections the nitrogen mustard induced acceleration appeared far more obvious—5 out of 10 of this group of animals revealing significant though small amounts of spleen amyloid

In the various control groups which, apart from casein received no further treatment than saline injections no amyloid occurred until after 18 days of casein at which stage 4 out of 10 animals were found to have developed slight to moderate spleen amyloidosis Unfortunately the control group receiving casein for 16 days was due to cannibalism reduced from 10 to 6 animals during the experimental period

Thus it seems that the most pronounced accelerating effect of nitro

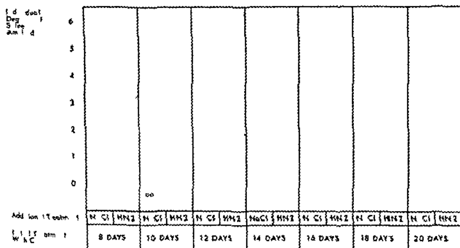


Fig 1

Individual degrees of spleen amyloidosis showing acceleration of amyloid formation in casein treated *mice with nitrogen mustard (HN2) Compared with casein treated* saline injected controls

gen mustard was achieved during the experimental period between 14 and 18 casein injections the accelerating effect after 20 casein injections being less marked as the controls in this group did show a comparable though less marked degree of amyloidosis

DISCUSSION

The results of the present experiment indicate as was to be expected in light of the two phase cellular theory that it is impossible to accelerate a casein induced mouse amyloidosis at any stage earlier than the end of the primary pyroninophilic phase that is after approximately 2 weeks of casein treatment

The most obvious reason for this seems to be that the alleged exhaustion of the immune mechanisms which apparently causes the change from the pyroninophilic to the P A S positive amyloid phase (*Teilum* 1964) needs at least 12–14 days to develop in our experimental system This is in agreement with several histological studies earlier performed in this laboratory (*Christensen* 1963) As the accelerating effect of nitrogen mustard is believed to rely on an acceleration of this exhaustion it seems reasonable that its accelerating effect is dependent on the existence of a certain level (or quality) of the activity of the immune apparatus—a level which apparently ... s 12–14 days of casein treatment to develop

In the face of a long standing casein t ... his exhaustion will set in spontaneously after 17–18 d... s judged by the time of appearance of spleen amyloid ... controls After additional

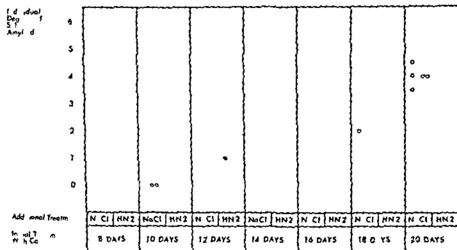


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In the face of a long standing casein treatment this exhaustion will set in spontaneously after 17–18 days (Fig 1) as judged by the time of appearance of spleen amyloid among the controls After additional

treatment of parallel groups with nitrogen mustard amyloidosis may be achieved as early as after 12–14 casein injections (Fig 1)

The Inversion Stage may thus be considered defined as the period between 12 and 16 days of casein treatment its duration being approximately 4 days Of course these figures can only be considered valid for the present experimental system which furthermore was confined only to the spleen Further it should be emphasized that any phase determination theoretically should be restricted to the single cell as the cells gradually become involved in the process—not necessarily simultaneously—and that such a determination on the whole organ only represents a rough statistical evaluation which however should be an allowed simplification

SUMMARY

The approximate duration and onset of the so called Inversion Stage between the initial pyroninophilic and the second amyloid phase of experimental mouse amyloidosis was determined

Groups of mice pretreated with casein for various lengths of time were subsequently injected with nitrogen mustard As controls failed to develop amyloidosis until after at least 18 casein injections while nitrogen mustard proved capable of accelerating amyloid formation in mice after 12 14 and 16 days of casein treatment it was concluded that the Inversion Stage in this experimental system covered approximately 4 days

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INFLUENCE OF ESTRADIOL ON UTERINE RENIN IN RABBITS

By

JENS BING and PETER CLAES ESKILDSEN

Received 4 vii 67

The reproductive organs of some mammals have been found to contain renin (*Stal emann* 1960 *Gross et al* 1964 *Bing & Farup* 1966 and *Ferris et al* 1967) The renin content of the rabbit uterus was found to increase in the second third of the pregnancy period and reach high values in the last third It remains high the first twenty hours or so after term and then rapidly falls to the non pregnancy values It was further found (*Bing et al* 1967) that the concentration of uterine renin is very low in immature rabbits the values rising from less than 0 5 to about 1 5 to 3 Goldblatt Units per g at an age of about 5 months when the animals weigh between 2 5 and 3 kg

As the pronounced changes in uterine renin concentration in non pregnant pregnant and post pregnant animals occur at the same time as changes are found in estrogen secretion it was the aim of the present work to study the relationship if any between estrogens and uterine renin content For this purpose the concentration of uterine renin was determined both in non treated and in estradiol treated castrated mature rabbits as well as in non treated and estradiol treated immature animals and in mature animals receiving very high doses of estradiol In some of these rabbits determination of the renin content of isolated endo and isolated myometrium as well as of the kidneys was performed The study further includes the demonstration of an angiotensin like pressor substance in extracts of uteri of immature and of castrated mature animals

MATERIAL AND METHODS

Albino rabbits of the strain from the State Serum Inst tut were used in most of the experiments The uterine and renal ti sue was weighed and frozen (—20 C) shortly after removal Biopsies of uterine tissue were performed by remo al of the supravaginal part of one of the t o horns

Isolation of endometrial and myomet al tissue was obtained by utting open flattening and freezing the uterus by placing it on a bra s plate on the top of a

Supported by grants from *King Christian X s Foundation* and the grant of *Re nholt W Jorck and Wife*

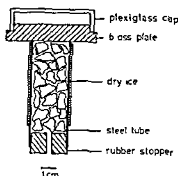


Fig 1

For the separation of isolated endo or myometrium an uterine horn was cut open and flattened on a brass plate which was placed on the top of a steel tub containing dry ice and surrounded by a plastic tube which fitted into the turning lathe In order to minimize the formation of frost the plate with the preparation was covered by a plexiglass cap before and after the turning

steel tube which was filled up with dry ice(Fig 1) and placed in a turning lathe In this way the frozen tissue could be turned either with the mucosa or the muscular coat facing the plate Before turning a representative strip of the frozen tissue was removed cut in a Pearse cryostate to sections about 8 μ thick stained and used for measurement of the width of the two layers the endo and myometrium The turning lathe could thereafter be adjusted to remove a little more than one of the layers leaving most of the other After this isolation procedure strips of the preparations were removed cut and stained so that the purity of th preparations could be determined

Extraction After repeated thawing and freezing the tissue was minced and extracted in a homogenizer with a pyrophosphate buffer (pH 5 3) containing 1 drop of toluene per ml After extraction for 4 to 24 hours the extract was centrifuged and stored at —20 C

Renin bioassays we e performed with the direct method when the extracts contained more than about 0 02 Goldblatt Unit per ml and with the indirect method when they contained less renin In both methods a highly purified hog renin preparation was used as a standard[1] The bioassays were performed on female albino rats weighing about 200 g anaesthetized with 20 mg of amytal and pretreated with 0 005 mg ergotamine tartrate or if used for angiotensin assay further pretreated with 7 5 mg of pentolinium tartrate The indirect assay was performed by determining the amount of angiotensin formed by incubation for 2 hours at 37 C of 100 μl of extract after dilution to a renin content below 0 03 Goldblatt Unit per ml with 100 μl of plasma from rats nephrectomized 24 hours previously In order to diminish angiotensinase activity both the extract and the plasma were subjected to a pH of 3 9 at 25 C for 20 minutes before use While the angiotensinase activity of the plasma was eliminated by this treatment the extracts had to be further diluted in order to escape this activity during the incubation The incubation mixture was covered by 0 6 ml of a mixture consisting of equal parts of toluene and paraffinoil (*Poulsen* 1957) After the incubation period 800 μl of trisbuffer pH 7 5 and 10 μl of 2 n HCl was added The tube was then placed in boiling water for 10 minutes the precipitate removed by centrifugation and 10 μl of 2 n NaOH added to the supernate which was stored at — 20 C

The estradiol preparation used was Ovex (Leo) containing 2 5 mg estradiol benzoate in 1 ml vegetable oil which in most cases was further diluted with sesame oil before use

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1 This preparation was kindly supplied by dr *Haas* and is identical with that donated by dr *Haas* to the WHO Laboratory for Biological Standards (Nat Inst Med Res Mill Hill London) from where also the standard angiotensin was obtained

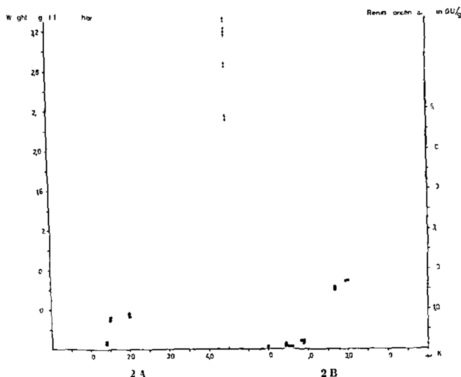


Fig 2 A and 2 B

The weight of one horn in g and the renin concentration in Goldblatt Units per g in immature and mature rabbits One horn weighing 5 9 g is not included in the figure

RESULTS

1 *Untreated Normal Immature and Mature Rabbits*

The influence of maturity on uterine weight and renin content is seen in Fig 2A and 2B which include some values given in a previous paper (1967) The figures show that the weight of one uterine horn as well as the renin content are very low in immature animals and are rising with increasing degree of maturity In mature rabbits the values both of weight and renin content scatter over a broad area independently of each other

2 *Castrated Mature Rabbits*

The effect of castration of mature rabbits is seen in Fig 3 which shows that castration is rapidly followed by a pronounced decrease in the uterine renin content The figure further shows that while values below 0 3 Goldblatt Unit per g were found until about 5 months after the time of castration some uteri removed between 1 and 6 months after the time of castration had a renin content between 1 and 2 Units per g

Contrary to the renin content the weight of the uterus decreased

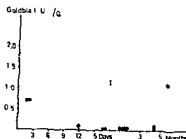


Fig 3

The renin concentration in Goldblatt Units per g in mature rabbits at different times after castration

slowly resulting in the finding of normal uterine weight but very low renin content in the two first weeks after castration In animals killed later than two weeks after castration there was so far a correlation between uterine weight and renin content as all animals with a renin content above 1 Goldblatt Unit had a weight of one horn that weighed more than 1 g while both values were below these limits in the rest of the animals

3 *Effect of Estradiol on Uterine Renin Content of Immature and of Castrated Mature Rabbits*

The effect of subcutaneously injected doses of 10 μg per kg of estradiol in oil per day in four days on *immature* rabbits weighing about 1 5 l g is seen in Fig 4A showing that the treatment resulted in a pronounced increase in the uterine renin content due to an increased weight and renin concentration these two values increasing independently

The control animals the values of which are also given in Fig 4A were either untreated or injected subcutaneously with the same sesame oil as was used for the solution of estradiol Any difference between the values from these two kinds of controls was not found

Three immature rabbits weighing about 1 4 kg were treated with as massive doses as 2 5 mg per day in 1 to 3 days This treatment resulted in the same pronounced increases in renin content the weight of one uterine horn and renin content being 0 13 g and 2 2 G U per g respectively after 24 hours 0 19 g and 1 8 G U after 48 hours and 0 74 g and 1 0 G U after 72 hours the total renin content thus being 0 29 0 34 and 0 74 G U after 1 2 and 3 days of treatment

Fig 4 B shows the result of estradiol treatment of *castrated mature* rabbits weighing from 2 6 to 3 7 kg while untreated the uterine renin content had been determined in a biopsy of one horn from some of these rabbits In this way it is clearly shown that treatment with doses of 5 to 10 μg per kg per day quickly normalizes both the renin concentration and the uterine weight resulting in the pronounced increase in renin content seen in Fig 4B Thus in one animal killed 24 hours after a single

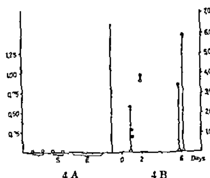


Fig 4

Fig 4 A The renin content in Goldblatt Dog Units of one uterine horn of non treated (N) sesame oil treated (S) which are both marked ○ and estradiol treated (E) immature rabbits (marked ●) The values from the immature rabbits who got massive doses of estradiol from 1 to 3 days are marked ⊗

Fig 4 B The results of estradiol treatment of castrated mature rabbits (marked ●) In some of the cases the renin content was studied in the same animal, while untreated (marked ○) The values from the castrated rabbits treated with massive doses estradiol are marked ⊗

injection of 5 μg per kg the weight of one horn rose from 0 5 to 0 9 g and the renin concentration from less than 0 03 to 2 5 Goldblatt Unit per g

Three of the castrated mature rabbits in which the typical decrease in uterine size was confirmed by laparotomy were treated for one to three days with massive doses of about 700 μg per kg per day With such large doses the weight of one uterine horn rose from 0 55 to 1 5 g the renin concentration from 1 8 to 4 7 G U per g and the total renin content of one horn from 1 1 to 7 0 G U

4 *Distribution of Renin in Endo and Myometrium*

In eleven normal mature rabbits in which the uterus was parted so that endometrial and myometrial tissue was obtained it was found that the endometrium in 6 cases contained from about 2 to 5 times as much renin as the myometrium the ratio of the remaining values in three cases being about 1 and in two about 0 4 In a limited number of non treated and estradiol treated castrated animals similar scattered values were obtained

5 *Effect of Estradiol on Renal Renin*

Contrary to the pronounced effect of estradiol on uterine renin the renin content of the kidneys was the same in untreated and estradiol treated immature rabbits with individual variations from about 8 to about 21 Units per g in both groups and with no differences in the weight of the kidneys

6 *Angiotensin like Pressor Substance in Extracts of Uteri from Immature and Castrated Mature Animals*

While the uterine extracts from mature rabbits gave a typical renin like pressor effect some of the uteri from immature or castrated rabbits contained a substance which gave an angiotensin like pressor curve This substance which could be found in concentrations corresponding to about 200 ng angiotensin per g was termostabile resisting boiling for 10 minutes Further identification of the substance has not yet been performed

DISCUSSION

It is well known that estrogens control the biosynthesis of ribonucleic acid and proteins including enzymes in the uterus The present study shows that uterine renin is influenced in this way the renin content decreasing after castration and increasing after estradiol treatment both of immature and castrated mature rabbits The studies do not give any answer to the question whether renin is formed both in endo and myo metrium The fact that normal values for uterine weight and uterine renin content were observed in some of the rabbits if studied more than 1 month after the time of castration speaks for an extra ovarian estrogen formation probably in the adrenals

SUMMARY

1 The uterine renin content is low in immature rabbits and increases when the animals come to maturity

2 Castration is rapidly followed by decrease in renin concentration while the decrease in uterine weight occurs more slowly In some castrated animals studied more than 1 month after castration normal values for both uterine weight and renin concentration were found

3 Estradiol treatment of immature and castrated mature rabbits re sults in increased renin content and uterine weight the effect on the renin content being more rapid than that on the weight

4 Renin is found both in endometrium and myometrium the ratio in most cases being from 1–5 but in a few cases below 1

5 Estradiol treatment is without effect on renal renin content

6 Extracts of uteri from immature and castrated mature rabbits con tain an angiotensin like substance

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PERICARDIAL ABSORPTION OF THORIUM DIOXIDE IN RATS

3 *Analysis of Thoracic Duct Lymph*

By

TROND KLUGE

Received 13 x 67

Studies on constrictive pericarditis in dogs (1) and human subjects (18) *suggest that lymph drained from the pericardium normally en ters the thoracic duct* Anatomical and roentgenological studies in rats (9 15) seem to support this theory

In a lymphangiographic study employing colloidal thorium dioxide (Thorotrast) the main lymphatic pathways from the pericardium in rats were visualized (9) Anastomoses from these lymphatics to the thoracic duct were suggested but could not be unequivocally demon strated

In order to obtain additional information concerning pericardial lymph drainage Thorotrast was applied in the pericardial cavity of rats The thoracic duct was drained and the radioactivity of the col lected lymph was measured The purpose of the study was

1 To investigate whether lymph from the pericardium collects in the thoracic duct
2 To observe whether this lymph enters the thoracic duct in its cranial as well as in its caudal portion
3 To study the rate of absorption from the pericardial cavity

MATERIALS AND METHODS

Thorotrast (Fellows Testagar Detroit Michigan USA) This is a sterile filtered dispersion of colloidal thorium dioxide It contains 24–26 per cent radioactive *thorium dioxide* (W/V) in a solution of 25 per cent dextrin and 0 15 per cent methylparaben 0 3 ml of this preparation was injected into the pericardial cavity of each animal

Collection of Lymph

PORTEX nylon cannulas (Portland Plastics Ltd Hythe Kent England) were used for cannulation of the thoracic duct Two calibers were employed 18 gauge with O D 0 63 mm and 17 gauge with O D 0 75 mm The inner plastic bag surround ing each catheter was used for collection of lymph Prior to use it was ensured that catheters and bags did not give radioactive emission They were than flushed with a 1 6 saline dilution of heparin 50 microg/ml (Upjohn Kalamazoo Michigan USA)

Experimental Procedure

A total of ?4 adult male rats weighing 250–350 g were used for the experiments They were anaesthetized with ether/alcohol (2 1) through an endotracheal tube (10) The chest was opened through a left thoracotomy and Thorotrast was injected into the pericardial cavity The chest wall was closed and the thoracic duct was immediately catheterized in the neck by the technique of *Saldeen & Linder* (17) Once introduced the catheter could be passed distally as the rat thoracic duct does not possess valves (16) As experienced by previous authors (3 17) the duct allowed the introduction of a catheter somewhat larger than the duct itself Hence it was ensured that no free space existed between the catheter and the wall of the thoracic duct

In 10 animals the tip of the catheter was placed in the cranial segment of the duct central to the l mpho venous junction In 10 other animals the catheter tip was positioned in the caudal thoracic segment just above the level of the diaphragm (Fig 1) The catheters were connected with the collecting bags and were fixed to the neck muscles and to the skin The tracheostomy and neck incision were closed and the animals were placed in restraining cages They were allowed a standard diet (Felleskjøpet Oslo) and free amounts of water during the experimental period Lymph was collected in different bags 1 ? 4 f 8 12 16 20 and 24 hours after the injection The volumes in the bags were measured and the lymph was transferred to heparinized plastic tubes at 4 C At termination of the experiment the position of the catheter was controlled during autopsy In two additional animals subjected to pericardial injection and lymph drainage 0 2 ml of blood were obtained at the same time intervals The blood was aspirated from the tail vein into a syringe containing 1 8 ml of heparin in saline (1 6) Two animals were sham operated i e lymph drainage was established without pericardial injection of tracer substance Lymph was collected at the same time intervals and studied as in the experimental animals

Counting of Radioactivity

Portions of 2 ml were taken from the lymph pools collected at different time intervals If the total volume in a pool was below 2 ml 1 ml of lymph was diluted with 1 ml of normal saline The contents were placed in a scintillation counter with a well crystal and counted at an energy range of approximately 350 keV At least 1000 counts were recorded for each sample *The background activity averaged 50 cpm*

RESULTS

Five of the animals in the two experimental groups were discarded owing to deficient lymph flow In three animals this was caused by displacement of the catheter in two obstruction by clotting of lymph was responsible In the latter X ray examinations revealed a distended cisterna chyli and leakage of contrast medium into the peritoneal cavity (3)

The results are based on the remaining 15 animals nine in which the catheter was in the cranial position (H) and six in which the catheter was in the caudal position (L) (Fig 1)

Lymph Flow

In both groups the total output of lymph per animal during 24 hours was approximately 30 ml The volume drained per hour was highest during the first 2 hours following cannulation with an average of about 3 ml per hour From 2 to 8 hours the flow approached a level of 1 5–2 ml per hour and then gradually decreased (Tab

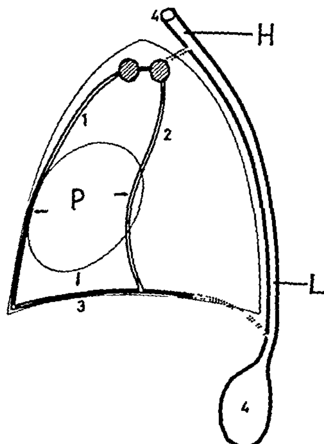


Fig 1

The main pathways in pericardial lymph drainage and their relations to the thoracic duct Lateral view P Pericardium 1 Parasternal lymphatics 2 Mediastinal lymphatics 3 Diaphragmatic lymphatics 4 Thoracic duct The levels of suspected anastomoses with the thoracic duct are indicated by interrupted lines The draining catheters were placed in the cranial (H) or in the caudal (L) portion of the duct

TABLE 1

The Mean Values of Lymph Flow (ml) and Radioactivity (cpm/ml) at Each Time Interval For Each Figure ± 2 s e Are Indicated

Hours	Mean volume of lymph		Mean cpm/ml	
	H ± 2se	L ± 2se	H ± 2se	L ± 2se
0–1	2 9 ± 0 2	2 8 ± 0 2	83 ± 30	20 ± 18
1–2	3 0 ± 0 2	3 0 ± 0 3	173 ± 59	32 ± 12
2–4	3 8 ± 0 8	4 4 ± 1 2	213 ± 74	48 ± 18
4–6	4 0 ± 0 8	3 4 ± 0 5	215 ± 59	43 ± 27
6–8	3 6 ± 0 6	3 7 ± 0 5	226 ± 74	38 ± 14
8–12	4 2 ± 0 8	4 4 ± 1 4	181 ± 61	52 ± 34
12–16	3 3 ± 0 6	3 0 ± 0 5	146 ± 32	55 ± 26
16–20	2 8 ± 0 4	2 7 ± 0 1	114 ± 45	52 ± 29
20–24	2 4 ± 0 3	2 6 ± 0 2	61 ± 31	30 ± 35

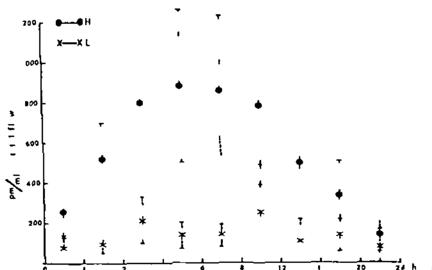


Fig 2

The total amount of radioactivity at different time intervals (see text) H and L indicate cranial and caudal position of the catheter For each mean value ± 2 s e (standard error) are indicated by a vertical bar

Radioactivity

The mean values (cpm/ml) for samples from the 9 H animals and the 6 L-animals at each time interval are presented in Table 1 In the H groups a maximum was recorded between 2 and 12 hours while the L-values showed only moderate variations (Table 1) As will be seen from the standard error (s e) marked differences between the different animals at each time interval were present Nevertheless the minimum counts recorded in the H samples were always higher than the maximum counts in the L-samples As expressed by the mean values (Table 1) the difference between the H group and the L-group was significant at all time intervals up to 16 hours

No specific radioactivity was detected in the blood samples from the two animals subjected to pericardial injection and thoracic duct drainage Analyses of the lymph from the two shamoperated animals also were negative *i e* they did not show radioactivity above the background level

Counts Related to Total Lymph Flow

The figures for cpm/ml were multiplied with the corresponding lymph output at each time interval These data give an expression of the total radioactivity and are presented in Fig 2 A maximum of radioactivity appeared between 2 and 12 hours in the H group while variations in the L-group were minor

DISCUSSION

Thorotrast is considered a reliable tracer when lymphatics are to be investigated (19) It does not leak from lymph capillaries (2) has a low toxicity in short term experiments (4) and its removal rate is not influenced by ether narcosis (6) Thorotrast has previously been used in studies on peritoneal lymph drainage (16) but not for investigations on the pericardium

In the present study only two animals were discarded owing to clotting of lymph in the catheter This low incidence may be due to the employment of heparin (3 12) and to the anticoagulant properties of Thorotrast (7 14)

The lymph flow rate per hour was highest during the first two hours after Thorotrast injection it remained constant at a lower level during the next six hours and then gradually decreased in the following period These observations correspond well with those of *Olin & Saldeen* (16) who used a similar technique In animals drinking a saline dextrose potassium solution (12) outputs up to 100 ml in 24 hours have been recorded

The flow rate did not differ significantly with the position of the catheter In the L-position the catheter probably occludes the anastomoses to the cranial segments of the duct (Fig 1) Hence one might suspect that a certain portion of the lymph be diverted from the cranial route and drain through the caudal anastomoses However the interposition of lymph nodes and valves (9 15) speaks against this possibility It seems more likely that the amount of lymph drained through anastomoses at both levels is low as compared to the total drainage through the thoracic duct which collects lymph from the entire intestinal tract the pelvis and lower extremities (15 16)

The presence of radioactivity in all lymph samples shows that lymph is drained from the pericardium through the thoracic duct It seems likely also that the thoracic duct is the main recipient of pericardial lymph drainage

The absence of radioactivity in the blood of the control animals does however not exclude the possibility of other lympho venous communications First blood from the tail vein has passed through the reticulo endothelial system where most Thorotrast particles are retained (7 13) Second weak emission from the small blood samples may have been lost in the background activity

The significant difference between the two experimental groups during each time interval permits the following conclusion Lymph is drained from the pericardium both through a cranial route via the parasternal/mediastinal lymphatics and through a distal route via the diaphragmatic lymphatics as indicated in Fig 1 This conclusion is in good accordance with earlier anatomical (15) and lymphographic (9) studies The present study also indicates that more pericardial lymph

is drained through the cranial than through the caudal anastomoses. This agrees with the findings of *Olin & Saldeen* (16) who stated that only a minor portion of diaphragmatic lymph enters the thoracic duct.

Radioactivity was detected in lymph samples already during the first hour of drainage (Table 1). In an electron microscopical study (8) most of the injected thorium dioxide was absorbed through the mesothelium during the first hour. By lymphography, however, visualization of lymphatics was delayed until the second hour (9). This difference between results obtained by lymphography and radioactive analyses is probably due to the greater sensitivity of the latter method.

The concentration of radioactivity in the lymph samples increased during the first 2 hours and attained a maximum between 2 and 12 hours. Studies on absorption from other serous cavities (5, 11) also indicate that an equilibrium is established in 1–2 hours and lasts for longer periods of time.

SUMMARY

Lymph drainage from the pericardium was studied by injection of Thorotrast into the pericardial cavity of adult rats. The thoracic duct was cannulated and lymph was collected from the cranial or from the caudal portion of the duct. Samples were collected at different time intervals and analysed in a well scintillation counter. Radioactivity was present in lymph samples already 1 hour following pericardial injection and reached a maximum between 2 and 12 hours later.

The results indicate that lymph drainage from the pericardium enters the thoracic duct in the cranial as well as in the caudal portion of the duct. More pericardial lymph was drained through the cranial than through the caudal anastomoses.

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A MEMBRANE FILTER METHOD FOR THE DEMONSTRATION OF BACTERIA BY THE FLUORESCENT ANTIBODY TECHNIQUE[1]

4 *Experimental Studies of the Demonstration of* Shigellae *in Water from Various Sources*

By

DAN DANIELSSON and GUNNAR LAURELL

Received 21 vi 67

The application of the membrane filter (MF) method in combination with the fluorescent antibody (FA) technique for the demonstration of bacteria in water was described earlier (2 3) After water had been filtered through a non fluorescent MF the latter could then be stained directly with FA for subsequent examination with a fluorescence microscope This technique allowed a specific bacteriological diagnosis within 2 hours and also a quantitation of the bacteria in question if the number of organisms was at least 1 000 per litre of filtered water (2) The sensitivity was increased by allowing organisms trapped on an ordinary white MF to multiply in nutrient broth and then filtering the broth through a non fluorescent MF for subsequent FA staining This technique named the two step procedure allowed the detection of bacteria within 4–6 hours at concentrations of 2–50 organisms per litre of filtered water The same sensitivity was arrived at with a corresponding enrichment procedure for 14–18 hours followed by FA staining of ordinary smears prepared from the sediment of centrifuged broth (3) These 3 modifications were successfully employed for a rapid demonstration and quantitation of enteropathogenic *Escherichia coli* in accidentally contaminated drinking water (4)

It is known that shigellosis can be an important waterborne disease Therefore it was of interest to test whether the MF methods in combination with FA techniques also could be used to demonstrate Shigellae in water The present paper presents a study of the specificity of fluorescein conjugated anti Shigellae globulins and the results of the application of the combined MF method and FA technique for the demonstration of Shigellae in experimentally infected and naturally polluted water of diverse origins

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examined for the coliform density with the standard multitube method (MPN method) according to current techniques (1) and for *Shigellae* with MF techniques in combination with FA methods as described below Water samples that gave positive FA tests for *Shigellae* were cultured on Conradi Drigalski blue agar plates before and after they had been enriched in broth (see below) Lactose negative colonies (at least 15–20 colonies) were studied further with biochemical tests (mannitol positivity lactose sucrose negativity indol negativity and urease negativity) and subsequent agglutination tests with polyvalent anti *Shigellae* sera after suspicious colonies had been reinoculated on blue agar plates

River water Water samples were collected from the river Fyris at 3 different stations (stations A B C) and examined in the same way as described above

Filtration Procedures Handling of MF and FA Staining

Water samples of 1 litre were filtered through

1) Black non fluorescent MF (Millipore HAB(P)G 047) From these filters circular pieces were stamped out with a diameter of 12 5 mm They were stained with FA as described before (2) When river water was used it was possible to pass only 250–500 ml of water through the filters

2) White MF which were put into 30 ml of nutrient broth and incubated at 37 C After 6 hours enrichment two 10 ml samples were passed through another black non fluorescent MF (Millipore HAB(P)G 025) and these were treated as described above This technique is called the two step procedure (3) The remaining broth was incubated for another 16–18 hours 4 5 ml of the latter was centrifuged for 20 min at 3 000 r p m the supernatant was decanted and the sediment suspended in saline Ordinary smears were prepared on slides and subsequently stained with FA in the usual way

Fluorescence Microscopy

A Zeiss fluorescence microscope equipped with a dark field condensor and an Osram HPO 200 mercury lamp was used A BG 12 (3 or 4 mm) was used as primary filter and a Zeiss 47 or a Zeiss 50 either alone or in combination as secondary filters The microscope was fitted with Zeiss equipment for incident illumination for the inspection of FA stained non fluorescent MF as described elsewhere (2) Ordinary smears were examined in the usual way

RESULTS

FA Staining Properties of FITC Labelled anti Shigellae Globulins before and after Absorptions

FA staining of Shigellae The conjugate of anti *S dysenteriae* serotype 1 gave a 3–4+ reaction with its homologous strain (Table 1) No reactions were observed with other *Shigellae* strains In the corresponding way the conjugates of anti *S flexneri* serotype 1 and anti *S boydii* serotype 1 gave 3–4+ reactions only with their homologous strains *S flexneri* serotype 3a however also gave a 1–2+ reaction with the conjugate of anti *S flexneri* serotype 1

The polyvalent conjugates of anti *S dysenteriae* (covering the serotypes 1–8) of anti *S flexneri* (1–6 x variant and y variant) of anti *S boydii* (covering serotypes 1–11) and of *S sonnei* I and II gave 3–4+ reactions only with the strains within their corresponding groups

FA staining of Escherichia coli *and* Alcalescens dispar The polyvalent conjugates of anti *S dysenteriae flexneri boydii* and *sonnei* were tested against the international strains of *Escherichia coli* representing the serotypes 01–0147 and against the strains of Alcalescens dispar

TABLE 1

FA Staining Reactions Obtained when FITC Labelled Anti Shigellae Globulins (Monovalent and Polyvalent as Indicated) were Tested at their Working Dilutions with Different strains of Shigellae

Strains of *Shigellae* tested	FITC labelled anti *Shigellae* globulins						
	S dysenteriae (serotype 1) Test dil 1 20	*S dysenteriae* (serotypes 1–8) Test dil 1 10	*S flexneri* (serotype 1) Test dil 1 10	*S flexneri* (serotypes 1 6 x y) Test dil 1 30	*S boydii* (serotype 1) Test dil 1 15	*S boydii* (serotypes 1–11) Test dil 1 10	*S sonnei* (serotypes I & II) Test dil 1 20
S dysenteriae 1	3–4+	3–4+	–	–	1+	–	–
S dysenteriae 2 3 4 5 6 7 8	–	3 4+	–	–	–	–	–
S flexneri 1a	–	–	3–4+	3–4+	–	–	–
S flexneri 2a	–	–	–	3–4+	–	–	–
S flexneri 3a	–	–	1–2+	3–4+	–	–	–
S flexneri 4a 5a 6a x var & y var	–	–	–	3–4+	–	–	–
S boydii 1	–	–	–	–	3–4+	3–4+	1+
S boydii 2 3 4 5 7 8 9 10 & 11	–	–	–		–	3–4+	–
S boydii 6	–	–	–	–	–	3–4+	1+
S sonnei I & II	–	–	–		–	–	3–4+

representing the serotypes 01–08. Strains cross reacting with anti Shigellae globulins are listed in Table 2. It will be seen that no cross reactions were observed between the anti *S. sonnei* conjugate and the tested strains. The anti *S. dysenteriae* conjugate gave 3+ or 3–4+ reactions with *Escherichia* coli 038, 0121, 0124 and 0130 and 1–3+ reactions with 014, 058 and 0112ac. The anti *S. flexneri* conjugate gave 3–4+ reactions with *Escherichia coli* 013 and 0147 and 2+ reactions with *Escherichia coli* 019 and 0129. The anti *S. boydii* conjugate gave 3–4+ reactions with *Escherichia coli* 053 and 0105 and 1–3+ reactions with 01, 03, 014, 039, 0102 and 0143. No cross reactions were observed between the conjugates and the other strains of Escherichia coli and the strains of Alcalescens dispar.

TABLE 2

FA Staining Reactions between Polyvalent anti Shigellae *Conjugates and Strains of* Escherichia coli *Representing O Groups 1 through 147 and Strains of* Alcalescens Dispar *Representing O Groups 1 through 8*

Strains tested	FITC labelled polyvalent anti *Shigellae globulins*			
	S. dysenteriae (serotypes 1–8) Test dil 1: 10	*S. flexneri* (serotypes 1–6, x, y) Test dil 1: 30	*S. boydii* (serotypes 1–11) Test dil 1: 10	*S. sonnei* (serotypes I & II) Test dil 1: 20
E. coli 01	–	–	2–3+	–
03	–	–	1–2+	–
013	–	3–4+	–	–
014	1–2+	–	1–2+	–
019	–	2+	–	–
038	3+	–	–	–
039	–	–	2+	–
053	–	–	3–4+	–
058	2–3+	–	–	–
0102	–	–	1–2+	–
0105	–	–	3–4+	–
0112ac	2–3+	–	–	–
0121	3+	–	–	–
0124	3+	–	–	–
0129	–	2+	–	–
0130	3–4+	–	–	–
0143	–	–	1–2+	–
0147	–	3+	–	–
Alcalescens Dispar 01–08	–	–	–	–

Absorptions of the polyvalent anti S. dysenteriae *conjugate.* With regard to the cross reactions observed, absorption experiments were required in order to obtain specific conjugates. Separate portions of the polyvalent anti *S. dysenteriae* conjugate were absorbed with *Escherichia coli* 038, 058, 0121 and 0130 and then tested against these strains and the individual strains of *S. dysenteriae*. The results are presented in Table 3.

It will be seen that absorption of the conjugate with *Escherichia coli*

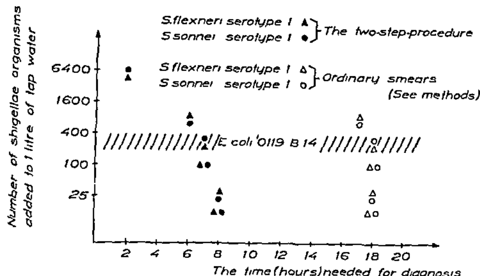


Fig 1

The time needed for the demonstration using fluorescent antibodies of vari us numbers of different serotypes of *Shigellae* organisms in experimentally contaminated tap water

from sediments of enriched broth The addition of an unrelated strain Escherichia coli 0119 B14 in concentrations of 200–400 organisms per litre did not interfere with possibilities of detecting Shigellae organisms

Examination of River Water and Well Water with Regard to Coliforms According to MPN Procedures and with Regard to Shigellae *with the Use of the Combined MF Technique and FA Method*

Water samples were collected from 3 different places of the river Fyris and from 10 different wells The results of these investigations are presented in Table 6

It will be seen that the river water was heavily contaminated with coliform bacteria Directly FA stained non fluorescent MF through which river water had been passed could not be examined owing to the occurrence of numerous coarse particles With the use of the two step procedure and ordinary FA stained smears organisms were stained with conjugates against *S flexneri sonnei, dysenteriae,* and *boydii* The same results were obtained with unabsorbed as well as absorbed conjugates No *Shigellae* could however be isolated by conventional methods

The coliform density in two of the wells was 500 thermolabile and 10 respectively 80 thermostable coliforms per 100 ml of water Examination of the water from these wells with directly FA stained non fluorescent MF gave negative results After enrichment of the water however organisms were stained with conjugates against *S flexneri* and *S dysenteriae* with the two step procedure as well as with FA

TABLE 6

Results from the Examinations of River Water and Well Water with MPN Procedures and the combined MF Method and FA Technique

Source of water	Coliform density per 100 ml (MPN test)		Results of FA tests with polyvalent anti *Shigellae* conjugates (unabsorbed and absorbed)		
	35 C	45 C	Direct FA staining of non fluorescent MF	FA staining of non fluoresc MF after enrichment (Two step procedure)	FA staining of ordinary smears after enrichment
The Tyris (Place *A*)	150 000	8 000	Illegible	*S flexneri dysenteriae boydii & sonnei*	*S flexneri dysenteriae boydii & sonnei*
The Tyris (Place B)	30 000	5 000	Illegible	*S flexneri dysenteriae boydii & sonnei*	*S flexneri dysenteriae boydii & sonnei*
The Tyris (Place C)	5 000	800	Illegible	*S flexneri & boydii*	*S flexneri & boydii*
Well waters 1 & 2	500	10 & 80	Negative	*S flexneri & dysenteriae*	*S flexneri & dysenteriae*
Well waters 1 10	0 240	0 17	Negative	Negative	Negative
Number of hours for diagnosis			1 – 2	7 – 8	18 – 20
See methods					

stained ordinary smears No *Shigellae* could however be isolated by conventional methods Water examined from the other 7 wells gave negative results with directly FA stained non fluorescent MF as well as with the two step procedure and FA stained ordinary smears after enrichment The water from these wells had low *coliform* densities

DISCUSSION

Difficulties involved in attempts to prepare specific fluorochrome labelled polyvalent anti *Shigellae* globulins have been reported by *La Brec et al* (5) and by *Thomason et al* (9) In the present paper antigens prepared by the Roschka method were used for immunization (8) This seemed to result in immunofluorescent conjugates quite comparable as to specificity with those obtained by *Thomason et al* (9)

When testing the polyvalent anti *Shigellae* conjugates against 147 international serotypes of *Escherichia coli* and eight serotypes of *Alcalescens dispar* the anti *S sonnei* conjugate showed complete specificity The other three polyvalent anti Shigellae conjugates showed however cross reactions with several different serotypes *of Escherichia coli* This is in agreement with the results obtained by *Thomason et al* (9) Certain differences were however noted The conjugates used by these authors gave also reactions with *Escherichia coli* 04 018 032 079 083 and 0135 which was not the case in this investigation On the other hand we observed strong reactions with *Escherichia coli* 013 and 0147 The latter strain was not however included by *Thomason et al*

There can be several *explanations of* these *differences* The conjugates used by *Thomason et al* covered a few more serotypes than ours On the other hand the working dilutions of our conjugates were higher than those used by these workers and a higher dilution usually increases the specificity by eliminating some of the non specific reactions Finally the technique used for the preparation of antigens for immunization might have been of importance since K antigens may occur in some serotypes of *S dysenteriae, S flexneri S boydii* and *S sonnei (9) The presence of* these K antigens can be expected to intensify non specific reactions It is not known however whether the Roschka technique for the preparation of antigens completely eliminates K antigen(s) but probably it diminishes the risk of production of K antibodies

The cross reactions between the polyvalent anti *Shigellae* conjugates and *Escherichia coli* could be eliminated by absorbing the conjugates with these strains *It was found* that certain strains had better absorbing capacity *than others The* absorptions had however some disadvantages Thus the conjugates had lower FA titres after absorption and the staining reactions *were greatly* reduced in intensity with some of the individual *Shigellae* serotypes A possible way *to solve this pro*

blem would be to supplement the polyvalent conjugates with these individual serotypes

The polyvalent anti *Shigellae* conjugates could be successfully used in combination with MF procedures for a rapid demonstration of *Shigellae* organisms in experimentally infected tap water A specific diagnosis was arrived at within 2 hours at concentrations of about 1 000 *Shigellae* organisms per litre of water and with the use of an enrichment procedure called the two step procedure within 6–8 hours at concentrations of about 5–50 organisms per litre These results are in complete agreement with reported results obtained with enteropathogenic *Escherichia coli* (2 3 4)

The experiments with naturally polluted waters have been less promising With heavily polluted water such as that from the river Fyris non fluorescent MF for direct FA staining could not be used because too many coarse and dense particles were present With the use of the two step procedure organisms were stained with all the polyvalent anti *Shigellae* conjugates used unabsorbed as well as absorbed No *Shigellae* could however be isolated by culture This might be an expression of the higher sensitivity of the FA method but for several reason it is more likely that it was a false positive reaction One reason is that diarrhoea caused by Shigellae has been extremely rare in Sweden within the last ten years It would therefore be surprising if many types were found although any clinical cases had not occurred in the community Efforts to isolate *Shigellae* were also unsuccessful

It was of interest to note that non specific FA staining reactions were not observed when well waters with coliform densities lower than 300 thermolabile coliforms per 100 ml of water were examined These results suggest that immunofluorescent techniques combined with MF methods could be of value if less heavily contaminated water sources were to be checked for example in the case of drinking water accidentally contaminated with sewage water or in the case of biological warfare when a preliminary diagnosis is promptly required For the time being however the experiments are too limited to permit more general conclusions or recommendations

SUMMARY

Fluorescein labelled polyvalent anti *Shigellae* globulins were prepared for *S dysenteriae S flexneri S boydii* and *S sonnei* If tested against 147 serotypes of *Escherichia coli* and eight serotypes of *Alcalescens dispar* the anti *S sonnei* conjugate showed complete specificity while the others gave cross reactions with several serotypes of *Escherichia coli* The cross reactions were removed by absorptions This resulted in a reduction of the staining reactions with some of the individual Shigellae serotypes After these investigations of specificity the reagents were used in combination with membrane filter (MF) methods

for the detection of *Shigellae* organisms in water of diverse origins Using experimentally infected tap water *Shigellae* organisms were demonstrated within 2 hours at concentrations of 1 000 organisms per litre and within 6–8 hours at concentrations of about 5–50 organisms per litre If heavily polluted river water and well water with coliform densities of more than 500 coliforms per 100 ml of water were examined organisms were stained with all the polyvalent anti *Shigellae* conjugates used unabsorbed as well as absorbed The results were regarded as false positive reactions Any non specific reactions were not observed when well water with coliform densities lower than 300 coliforms per 100 ml of water was examined The possibility of using immunofluorescent techniques in combination with MF methods with a view to checking less heavily contaminated water sources for entero pathogenic bacteria has been discussed

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INVESTIGATIONS ON THE ENZYMES AND TOXINS OF STAPHYLOCOCCI

Assay of Lipase Using Tween as the Substrate

By

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The property of coagulase positive staphylococci to cause opacity in egg yolk was first described by *Gillespie & Alder* (4). It was shown by these authors that a good correlation existed between the egg yolk reaction and the capacity to hydrolyse lard. The reaction on egg yolk was therefore thought to be due to a lipase. Investigations by *Shah & Wilson* (10) as well as *Tirunarayanan & Lundbeck* (12) confirmed that the egg yolk reaction was caused by a lipase which acted on the lipoprotein lipovitellenin in egg yolk and liberated fatty acids. Analysis of the fatty acids revealed a higher content of palmitic acid than stearic or oleic acids (12). A similar observation concerning the relative amounts of liberated palmitic, stearic and oleic acids was made by *Vadehra & Harmon* (14) during studies on the action of staphylococcal lipase on milk fat. It has also been found that staphylococcal lipase liberates stearic, palmitic and oleic acids quantitatively from synthetic triglycerides independent of the position at which the fatty acids are substituted on the glycerol molecule (1).

The investigations so far performed dealt with the analysis of the end products of the reactions, whereas the rates of hydrolysis of the individual ester bonds were not very much investigated. *Drummond & Tager* (3) studied the hydrolysis of different triglycerides and found that only tributyrin was hydrolysed by the staphylococcal enzyme. On the other hand *Stewart* (11) observed that the lipase from staphylococci hydrolysed tributyrin as well as other triglycerides.

Certain difficulties were encountered while studying the effect of staphylococcal lipase upon the triglycerides of higher fatty acids because of their insolubility in water and the effect of their particle size on the rate of enzyme reaction. The different fatty acid esters of Tween therefore seem better suited for enzyme kinetic studies as they are readily miscible with water.

The usability of Tweens as substrates for lipase has previously been established. Thus *Hurlein & Pil* studied the determination of lipo

protein lipase (clearing factor) in human sera and found that the re activity was twice as great with Tween 80 as it was with Tween 60 (5) In the present study the action of staphylococcal lipase on the Tweens was studied by a spectrophotometric as well as by the agar plate assay method described previously (7)

EXPERIMENTAL

The identification of the lipoprotein lipovitellenin as the substrate and the fatty acids as the end products in the egg yolk reaction indicated that the enzyme involved was a lipase (10 12) The liberation of palmitic acid in amountts greater than either stearic or oleic acids (12) suggested that esters of palmitate were more readily attacked by the enzyme than esters of the two other acids In order to study this phenomenon further a number of experiments were performed in which the different fatty acid esters of Tween were used as the substrate

Lipolytic activity can be described as a hydrolytic cleavage of the ester bond between an alcohol and a fatty acid The enzyme reacts with the ester to produce an acyl derivative Under the usual experimental conditions the acceptor for such acyl derivatives is water Where the fatty acid is insoluble calcium or proteins can function as acceptor Thus under such conditions a measure of the calcium salt that is formed is proportional to the enzymatic reaction With Tween as the substrate no fatty acid, even if insoluble in water is precipitated in the absence of calcium There is no change either in the pH of the reaction mixture The precipitate which is formed in the presence of calcium is homogenous because of the surface active properties of Tween until the concentration of the fatty acid salt formed exceeds 300 μ Mol (5) Thus within this range of fatty acid concentration optical density measurement can be performed with good accuracy especially using a spectrophotometer with a light path of 0 001 mm

The spectrophotometric method was useful to study the rates of the reactions as well as to establish the requirement for optimal activity like for instance pH calcium and magnesium ions The agar plate assay method was convenient to use for routine testing of activity with unpurified supernatants

HYDROLYSIS OF THE TWEEN COMPOUNDS STUDIED WITH THE AID OF THE SPECTROPHOTOMETRIC METHOD

A Unicam spectrophotometer model SP 800 equipped with an automatic unit for repeated changing and recording of the absorption of four cuvettes together with their corresponding blanks was used The repetition of recording was set at 1 minute interval and the cells were maintained at 37 C by circulating water from a thermostated water bath through the cell holder The blanks contained all the components of the reaction mixture for the test solution except for the substrate which was replaced by buffer Unless otherwise stated the reaction mixture consisted of

0 1 ml 10 per cent Tween in 0 05 I Tris HCl pH 7 6
0 1 ml 1 M CaCl in 0 05 I Tris HCl pH 7 6
0 1 ml of culture supernatant as enzyme source or purified enzyme preparation
2 7 ml of 0 05 I Tris HCl pH 7 6

The Tweens were hydrolysed by the enzyme and the liberated fatty acids reacted with calcium to form insoluble salts which increased the

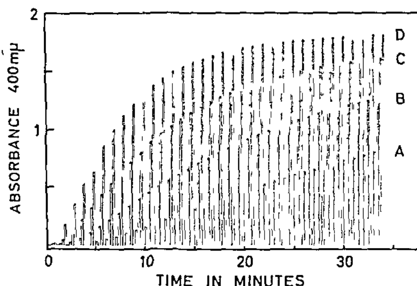


Fig 1

Spectrophotometric recording of the hydrolysis of Tween 20 by culture supernatant of Staph aureus 44 A = 3 μl B = 6 μl C = 12 μl D = 24 μl

turbidity with time The turbidity was measured at 400 mμ For the spectrophotometric studies the culture supernatant of Staph aureus of phage group III having the laboratory number 44 was used as the enzyme source The experiments were done with a preparation chromatographed on a column of Sephadex G 100 as described elsewhere (13)

Enzyme Concentration and the Rate of Hydrolysis

The effect of different amounts of the enzyme preparation on the hydrolysis of Tween was studied The spectrophotometric recording of the hydrolysis of Tween 20 showing a change in optical density at 400 mμ is shown in Fig 1 A final concentration of 3 6 12 and 24 μl of the enzyme solution was used The rate of formation of precipitate was high with 24 μl and declined with decreasing amounts of the supernatant The initial reaction velocity expressed as the increase in absorption at 400 mμ per minute (E_{400} per min) was plotted against the amount of the enzyme (Fig 4) A linear relationship was obtained

Effect of pH on the Hydrolysis of Tween

For the study of the effect of pH on the hydrolysis of Tween 20 appropriate buffer solutions were used 0 2 M Na acetate acetic acid buffer was used for the pH range 4 0–7 4 and 0 1 I Tris HCl buffer

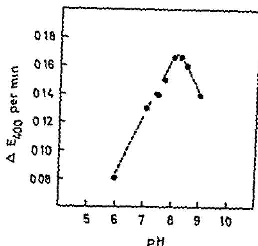


Fig. 2

Relationship between the pH and the rate of hydrolysis of Tween 20

was used for the pH range 7.4–9.0. Little activity was demonstrable below pH 6. The initial reaction velocity was plotted against the pH at which the reaction was studied (Fig. 2). It was observed that as the pH was raised from 6 the activity increased and reached a maximum between 7.8 and 8.2. Further increase in pH decreased the enzyme activity. Thus a pH between 7.8 and 8.2 was found to be optimal for the enzyme to hydrolyse Tween 20. A broader pH optimum between 7.4 and 8.6 was found for the hydrolysis of the other Tween compounds.

Effect of Calcium and Magnesium Ions on the Hydrolysis of Tween

The rate of hydrolysis of Tween 20 in the presence of varying amounts of calcium and magnesium ions (as chloride) was studied using 6 μl of the enzyme preparation. No enzyme activity was demonstrable in the absence of either of these ions as no precipitate or change in pH occurred. At the concentration of 3.3×10^{-5} M or less of calcium there was no reaction. Increasing the concentration of calcium resulted in a gradual increase in the initial reaction velocity reaching a maximum between 3.3×10^{-3} and 1.0×10^{-2} M. Further increase in calcium concentration decreased the reaction velocity (Fig. 3). The activity of the enzyme in the presence of magnesium was similar to that of calcium but higher concentrations of magnesium were required. No activity was demonstrable with magnesium up to a concentration of 3.3×10^{-3} M. With further increase the activity increased rapidly and reached a maximum around 7.0×10^{-2} M. Still higher concentrations did not affect the enzyme activity. Thus magnesium was found to be less active than calcium as fatty acid acceptor during hydrolysis of Tween 20.

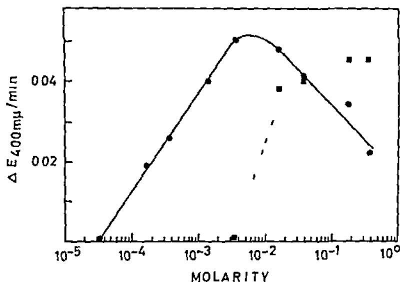
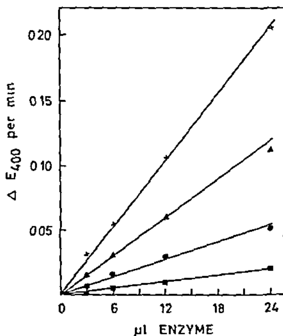


Fig 3

Effect of calcium (solid line) and magnesium (broken line) on the hydrolysis of Tween-20



F[illegible] 4

Relationship of e[illegible]ym[illegible] t[illegible] t[illegible] th[illegible] rates of hydrolysis of *different* Tween c[illegible]mpound[illegible]

×——× Twe[illegible]n[illegible]0 ▲——▲ Tween-40 ●——● Tween-60 ■——■ Tween-80

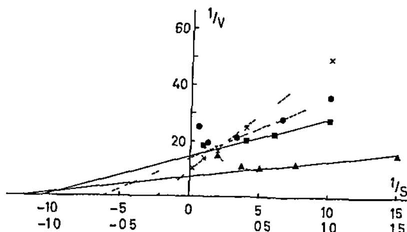


Fig 5

Lineweaver Burk plot for the evaluation of Michaelis constants for various Tween substrates Dotted and solid lines relate respectively to the high and low 1/s values on the abscissa

×——× Tween-20 ●——● Tween-40 ■——■ Tween-60 ▲——▲ Tween-80

Rate of Hydrolysis of Different Tween Compounds

A final concentration of 0 33 per cent of the Tween compounds as the substrate was found to be adequate to study the rates of hydrolysis in the presence of different amounts of the enzyme preparation A linear relationship was obtained when the initial reaction velocity was plotted against the concentration of enzyme with the four Tween compounds used (Fig 4)

Maximal activity was obtained with Tween 20 as the substrate and the reactivity with the other Tweens decreased in the following order

Tween-20 < Tween-60 < Tween-60 < Tween-80

The relative ratios of the reaction velocity as judged by the change in optical density with the different Tween compounds was 10 5 2 5 1 respectively

Effect of Substrate Concentration (s) on Reaction Velocity (v) and Evaluation of Michaelis Constants for Tween Compounds

The effect of different concentrations (between 1 0 and 0 003 per cent w/v) of the Tween compounds on the activity of lipase was studied For the evaluation of the theoretical velocity maximum (Vmax) and the Michaelis constant (Km) the values of 1/v and 1/s were plotted as shown in Fig 5 At high concentrations of the substrate there was no inhibitory effect on the reaction velocity by Tween 20 whereas the other Tween substrates showed inhibition From the plot the values for Vmax and Km were calculated

Substrate	*Vmax*	*Km (per cent)*	*Km (per cent)*
Tween-20	0.105	0.435	0.435
Tween-40	0.074	0.162	0.081
Tween-60	0.073	0.970	0.323
Tween 80	0.142	0.833	0.208

On the basis of proportional molecular weight

On the basis of weight the reactivity of staphylococcal lipase was the highest with Tween 40 and decreased in the following order Tween 40 < Tween 20 < Tween 80 < Tween 60 The numbers which follow the Tweens (T 20 T 40 etc) also represent the total number of oxyethylene linkages (9) Thus the number of such linkages increases proportionally the molecular weight of the Tweens Because the central core of the molecule together with the fatty acid form the minor part of the molecule it is concluded that the relative proportion of the molecular weights for T 20 T 40 T 60 and T 80 lie in the ratio of 1 2 3 4 *respectively This does not hold true for the polysorbates* which are also designated as Tween the differences in molecular weight being due to differences only in the weight of the fatty acids

STUDY OF THE HYDROLYSIS OF TWEEN COMPOUNDS WITH THE AGAR PLATE METHOD

Determination of Enzyme Activity by the Agar Plate Assay Method

The nutrient agar used had the composition described earlier (7) and was sterilized at 120 C for 30 minutes together with the Tween Unless otherwise stated 0 75 per cent Tween 60 was incorporated into the agar 0 02 per cent sodium methiolate was added to inhibit bacterial growth The pH was checked and adjusted to 7 8 before pouring the agar into Petri plates As the medium contained enough calcium and magnesium ions it was not necessary to add these co factors Prior to the incorporation in the agar the Tween compounds were treated with dry calcium oxide and filtered This removed any free fatty acids present in the preparations

To demonstrate the enzyme activity 0 15 ml of the culture supernatants was filled with micropipettes into 10 mm diameter and 3 mm deep holes cut in the agar The reaction was noted after incubation for 24 hours at 37 C Addition of more supernatant and further incubation for 24 hours increased the zone of reaction (Fig 6) Low activity was detected by filling the holes daily and incubating up to 4 days After this period the reactions were diffuse and not readily readable

Hydrolysis of Different Tween Compounds

In an earlier study (7) the reaction given by culture supernatants of staphylococci on egg yolk agar was studied Experiments with different concentrations of egg yolk showed that the best results were obtained with 0 75 to 1 25 per cent egg yolk Below this concentration

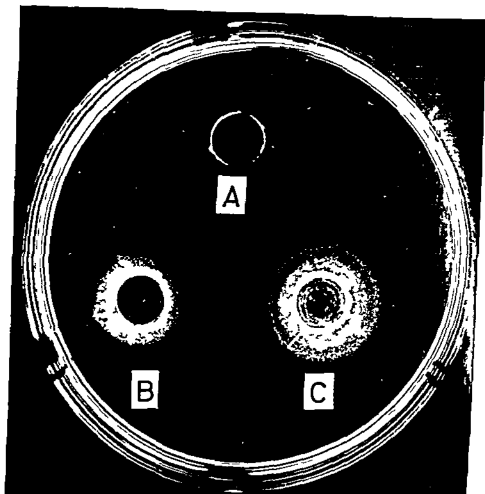


Fig 6

A = no enzyme control B and C = two concentrations of culture supernatant
Reaction given by culture supernatant of Staph aureus 44 on Tween-60 agar

the reactions were diffuse and with higher concentration they were difficult to read Tests with Tween compounds were performed in a similar manner

The concentrations of the Tween compounds used were 0 25 0 50 0 75 and 1 00 per cent The activity was tested using three concentrations of the culture supernatant (undiluted 1 10 and 1 100) The reactions were read daily after application of the enzyme for a period of up to three days (Table 1) No activity was demonstrable with Tween 80 as the substrate whereas precipitation occurred with the other Tweens The reactions given by culture supernatants with Tween 20 and Tween 40 were frequently diffuse and ill defined to permit accurate measurement of the zones of reactions On the other hand Tween 60 gave a bright zone with good demarcation and the results could be

read with ease and accuracy Thus for routine purposes the studies were done with Tween 60 as the substrate bearing in mind the limitations of the test due to the fact that its reactivity was lower than that of *Tween 20 or Tween 40 For his reason Horlein & Pilz* (5) also used Tween 60 although the reactivity was twice as great with Tween 80

TABLE 1
Hydrolysis of Tween Compounds Studied by the Agar Plate Method

Tween conc % (w/v)	enzyme concentration (diln)	Tween-20			Tween-40			Tween-60			Tween 80		
		zone of reaction in mm diameter after days											
		1	2	3	1	2	3	1	2	3	1	2	3
	undiluted	22	29	33 5	21	27 5	33 5	22 5	29	34 5	0	0	0
0 25	1 10	16	20 5	27 5	15	20	25	17	21	27	0	0	0
	1 100	0	13	20	0	14	16	0	15	20	0	0	0
	undiluted	22	30	32	21	28 5	35	22 5	29	34 5	0	0	0
0 50	1 10	16	21	26	15	19 5	24	17	21	26	0	0	0
	1 100	0	13 5	19 5	0	14	15	0	15	20	0	0	0
	undiluted	22	28	33	21 5	28	34 5	21 5	37 5	33	0	0	0
0 75	1 10	15	21	23	16	20	25	16	21	25	0	0	0
	1 100	0	13	17	0	13	15	0	13	17 5	0	0	0
	undiluted	20	28 5	33	21	28	33 5	20	26 5	30	0	0	0
1 00	1 10	15	19	26	16	20	24 5	14	19	23 5	0	0	0
	1 100	0	13	14	0	13 5	16	0	13	17 5	0	0	0

The lack of reaction of supernatants with Tween 80 as the substrate by the plate method was probably due to the lower concentration of the substrate or limiting amounts of calcium However cultures growing on such Tween-80 agar gave positive reactions The problem whether this is due to liberation of larger amounts of the enzyme by growing colonies or to their ability to split Tween at sites other than the ester linkage needs further study *Delmotte* (2) also found a low activity with Tween 80

Relationship of Enzyme Concentration to Formation of Precipitate

Although no great differences in the diameter of the zones of reactions were obtained with the different Tween substrates when using a single enzyme concentration there were differences when the enzyme content was varied (Table 1) It was thus apparent that the concentration of the enzyme played a greater role for the magnitude of the reaction than the type of substrate u d The effect of different concentrations of the enzyme was studied using 1 per cent Tween 60 as the substrate The enzyme solutions were f lled u ily for 4 days and read 24 hours after each filling Under th s n itions a linear re

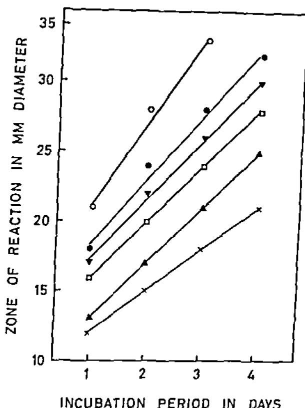


Fig 7

Reactions given by culture supernatants on Tween 60 agar plates Supernatants giving 21 mm (○—○) 18 mm (●—●) 17 mm (▼—▼) 16 mm (□—□) 13 mm (▲—▲) and 12 mm (×—×) after 24 hours incubation

lationship was obtained between the incubation period and the zone of reaction (Fig 7) These results were similar to those obtained with egg yolk as the substrate (7)

Hydrolysis of Tween 60 by Clinical Strains

Supernatants of cultures of 402 clinical strains grown for five days in Difco brain heart infusion broth and centrifuged at 20 000 g for 30 minutes at 4 C were tested for their activity to hydrolyse Tween 60 by the agar plate method Sodium merthiolate was added to the supernatants to a final concentration of 0 02 per cent to prevent bacterial growth The diameter of the zone of reaction obtained after repeated daily application for a period of four days was plotted against the number of strains giving the reactions 96 strains (24 per cent) did not give any activity (Fig 8) 72 strains (18 per cent) gave a low degree of activity (between 13 and 20 mm) 193 strains (48 per cent showed moderate activity *(from 21 to 30 mm) and 41 strains (10 per cent)* had high activity giving a reaction of more than 30 mm diameter

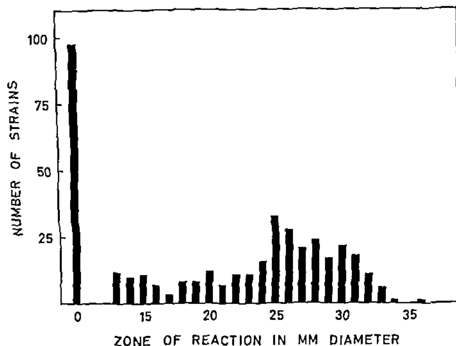


Fig 8
Lipase activity in the supernatants of 402 clinical strains

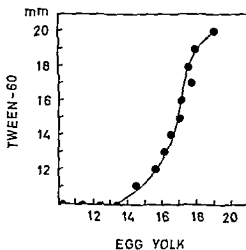


Fig 9
Comparison of the lipase activity given by supernatants of cl n al strains on egg yolk and Tween-60 agar plates

Thus 75 per cent of the strains gave a positive reaction on Tween 60 plates

Earlier studies using egg yolk as the substrate (7) with these 402 culture supernatants showed that 99 (25 per cent) had no activity while the rest gave various grades of activity Thus similar results were obtained with Tween and egg yolk substrates

Experiments with Tween 60 and egg yolk plates were also performed using the same culture supernatants of 402 strains which made a ready comparison possible The average values of the diameter of the zone of reaction with either substrate were plotted and an S shaped curve was obtained (Fig 9) Supernatants which showed activity lower than 14 mm on egg yolk agar plates did not give any reaction on Tween 60 plates It is obvious from the figure that the egg yolk reaction was more sensitive than the reaction on Tween 60

DISCUSSION

Earlier studies (4 7) demonstrated that staphylococci produced a factor which reacted on egg yolk giving clearance and precipitation This reaction was found to be due to a lipase which acted on the lipoprotein lipovitellenin of egg yolk and liberated fatty acids (10 12) The occurrence of lipase in the majority of clinical strains and its association with coagulase (4 6) suggested a possible relationship to the virulence of the bacteria A detailed investigation was therefore made on the properties of the lipase produced by staphylococci

Among the several substrates used for the study of the lipases there are few water soluble esters of higher fatty acids Triglycerides of stearic (C_{18}) or palmitic (C_{16}) acids are insoluble in water and could therefore not be used On the other hand the Tweens are miscible with water and therefore suited for such studies The relative rates of hydrolysis of the different Tween substrates at a concentration of 0 3 per cent showed that Tween 20 was hydrolysed at the maximal rate However evaluation of the Michaelis constants for the different Tween substrates gives a better insight into the reactivity of the lipase with the fatty acid esters Thus it was found that on the basis of their molecular weights the palmitate ester (Tween 40) was the most reactive followed in decreasing order by oleate ester (Tween 80) stearate ester (Tween 60) and laurate ester (Tween 20) By calculation the fatty acids released correspond to the proportion of 2 3 mol lauric acid 12 3 mol palmitic acid 3 1 mol stearic acid and 4 8 mol oleic acid Thus 61 per cent palmitic 15 per cent stearic and 24 per cent oleic acids would theoretically be released from an equimolar mixture of the fatty acid esters The results of the gas chromatographic analysis of the fatty acids isolated from egg yolk plates after reaction with the staphylococcal lipase indicated a content of 63 per cent palmitic 12 per cent stearic and 22 per cent oleic acids (12)

These studies support the earlier findings where a larger amount of palmitic acid than stearic or oleic acids was found to occur in the fat from egg yolk plates (12) and in milk (14) However these results are not supported by the findings of *O Leary & Weld* (8) *Weld Kean & O Leary* (15) *Weld & O Leary* (16) and *Shah & Wilson* (10) who obtained larger amounts of oleic than stearic or palmitic acid A care ful consideration of the experimental procedure reveals that these authors have directly extracted the fat with an organic solvent without preliminary acid treatment Thus they probably missed extracting the higher fatty acids which occur as calcium salts insoluble in organic solvents

SUMMARY

The activity of staphylococcal lipase was studied using the different fatty acid esters of Tween A spectrophotometric analysis showed that the enzyme had a pH optimum between 7 8 and 8 2 Between 3 3 × 10^{3} and 1 0 × 10 M calcium or 7 0 × 10 M magnesium were needed for optimal reaction with Tween 20 as the substrate The reactivity of the lipase with the palmitate ester (Tween 40) was the highest and it de creased in the following order Tween 80 (oleate ester) Tween 60 (stearate ester) Tween 20 (laurate ester)

The enzyme activity in the culture supernatants of 402 clinical strains was studied using Tween 60 as the substrate by the agar plate method Comparison of the activity on Tween 60 and on egg yolk agar plates indicated a good correlation between the two reactions The egg yolk reaction was found to be considerably more sensitive than the Tween 60 reaction

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MYCOBACTERIUM AVIUM

A Bacteriological and Epidemiological Study of M avium *Isolated from Animals and Man in Denmark*

Part 1 Strains Isolated from Animals

By

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Received 10 vii 67

This work is a bacterial study of *M avium* 196 strains isolated in Denmark from animals and 22 strains from Danish patients Forty one of the animal isolates and all the human isolates were serotyped (*Schaefer & Reggiardo* (7) *Schaefer* (5 6) *Bennedsen* (1)) and some distinctive characterization of serotype Avian II (43 strains) and of serotype Davis (7 strains) was possible The results obtained are a contribution to the species description of *M avium* the boundaries of which are in much dispute at present Furthermore the data elucidate important epidemiological problems such as the sources of infection and inter relationships of animal and human infections

The strains studied are all from Denmark They do not necessarily represent the world population of *M avium* Almost all the strains 205 out of 218 are from sources other than birds

The study is divided into two sections The first comprises the examination of strains isolated from animals Since these represent all the mycobacterial strains isolated during a certain period an indication can be gained of the types responsible for the manifest infection of the animals concerned On the basis of the examinations in the first part of the work a description is given of the typical pattern of *M avium* as it occurs in animals in Denmark as well as details concerning the few divergent results The second part (2) consists of strains isolated from man though limited to those with such a degree of virulence for rabbit and hen that on the basis of that finding and the results of other tests they were classified as *M avium* Each of these strains is compared with the typical pattern described in the first part of the work The second part concludes with a collective discussion of all the results

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The writers are grateful to Dr W B *Schaefer* for having serotyped the strains in question

MATERIAL AND METHODS

Material 196 strains were isolated from swine cattle poultry and other animals All except the 13 originating from poultry were received from Statens Veterinære *Serumlaboratorium*[1] during the period November 1961 to August 1963 as cultures on Lowenstein Jensen medium or in Besredka The strains were isolated from material sent to that laboratory for examination for mycobacteria When pathological processes are found in cattle such bacteriological examination is compulsory in Denmark while as regards the other animals it is voluntary

The material from hens and from chicken runs (soil manure) was collected from one district where special examinations were carried out by the Veterinary Directorate The organs were from diseased hens and the soil and manure specimens from chicken runs where the birds were infected with *M avium* All these strains were isolated at Statens Seruminstitut

TABLE 1

M avium *Animal Isolates*
Number of Specimens Divided According to Animal Group and Material from which the Mycobacteria Were Isolated

Animals	Specimens				
	Mesenteric gland	Other glands	Organs	Manure or soil	Total
Pigs	62	34	0	0	96
Cattle§	7?	12	0	0	84
Hens	0	0	8	5	13
Others†	1	2	0	0	3
Total	135	48	8	5	196

Some consisting of both glands and organs
§ 55 of these from calves and heifers
† Horse sheep mink

Table 1 shows the *distribution of strains according to animal group and kind of specimen* The majority of the strains from cattle originated from the mesenteric glands As regards the pigs many of the isolates were also from that localization *but mycobacteria were found quite frequently in the submandibular glands (recorded* under other glands)

Distribution according to county 58 pig strains were from Bornholm (an island in the Baltic isolated from the rest of Denmark) and all the 13 strains from hens were from another county (Holbæk) When these two sets of strains are excluded the remaining material from pigs and cattle was distributed evenly over the various counties this applies also to the serotyped strains The evaluation does not take into account the size of the animal population in the counties concerned

Serotyping was performed on 41 strains *Schaefer* examined 40 strains which had been sent to him on Lowenstein Jensen medium (6) These were 10 strains from pigs 14 from cattle 13 from hens and 3 from other animals Subsequently *Bennedsen* examined part of the same material and one further strain (from a heifer) using the method described by him (1)

Table 2 presents the distribution of the material according to serotype within the individual animal groups It will be seen that serotype Av II dominated only one *Av I strain being* found among the cattle isolates The strains of serotype Yandle (SSC 637) and Unclass special (SSC 812) isolated from cow and soil respectively (shown under cattle and hen in Table 2) failed as the only two out of the 196 *strains examined to show* definite alliance with *M avium* and therefore do not lie

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1 Thanks are due to Dr *H E Ottosen* Statens Veterinære Serumlaboratorium for the supply of this material

within the framework of the present investigation Thus they are omitted from the material in the subsequent part of the work and will be mentioned further only in the discussion

TABLE 2
M avium Animal Isolates
Number of Specimens Divided According to Serotype within the Individual Animal Groups

Serotype	Animals				Total
	Pigs	Cattle	Hens	Others	
Av I	0	1	0	0	1
Av II	10	10	12	2	34
Spont. aggl	0	1	0	0	1
Unclassified	0	2	0	1	3
Not tested	86	69	0	0	155
Yandle	0	1	0	0	1
Unclass special	0	0	1	0	1
Total	96(10)	84(15)	13(13)	3(3)	196(41)

Figures in brackets represent the total number of strains serotyped

Methods A detailed description of the techniques used and the system for registration of the results was given in a recently published paper (3) A survey of these methods is included in the present work in Tables 20 and 21 covering the *in vitro* and *in vivo* examinations respectively

All the experiments described subsequently were performed with actively growing cultures standardized to about 1 mg semi dried bacilli per ml (standard culture) or dilutions of it (3) On the basis of colony counts on Lowenstein-Jensen medium from a series of dilutions it was determined that 114 of the 120 strains examined in the *in vivo* experiments had 10^7 to 10^9 and six 10^6 to 10 viable units per ml The variations seemed to have no influence on the results For instance the above mentioned six strains killed all the hens in 18 to 56 days and all the rabbits in 14 to 27 days The bacterial doses used in the work will therefore be stated in milligrammes

All the strains were not subjected to all the examinations The number of strains examined by the different methods can also be seen from Tables 20 and 21 As regards the 120 strains included in the majority of the *in vivo* experiments 55 were from pigs 50 from cattle 12 from hens and 3 from other animals

All the 194 strains are included in the evaluation of the results to the extent to which they were examined by the various methods These form the basis for the description of the typical strain

Tuberculin tests with different sensitins were carried out with 19 strains only and therefore the results are not included in the present work

RESULTS

Evaluation of the experimental data In order to facilitate evaluation of the data collected not only from the point of view of the present work but also for use in other taxonomic studies all the results were punched on to IBM cards and tabulations were made on an electronic computer The results were evaluated for each animal group separately according to whether or not serology was performed However only minor irregularities were found between the animal groups as regards the distribution of results within the features examined For instance

in the rabbit experiments the strains isolated from cattle gave only slightly prolonged median survival times (3–4 days) as compared with those isolated from poultry and pigs No such displacement could be seen in the experiments on hens In consequence the source of the isolates was not taken into further consideration

Serotype Av II predominated among the serologically classified strains This will probably also apply to the other strains designated undetermined but the proportion is not known The results presented here are for two groups 1) the strains known to be Av II and 2) the undetermined strains comprising Av I *spontaneously ag* glutinable and unclassified strains and those not tested serologically

On account of the uniformity of the findings some of the results are given for both groups together

Colony morphology on Lowenstein Jensen and oleic acid albumin agar media was examined on 153 strains generally after three weeks using well isolated colonies Where growth on Lowenstein Jensen medium was typical the colonies were dysgonic and dome shaped with shiny smooth surface On agar medium the colonies were small and flat with somewhat irregular edge and undulated surface greyish white, quite translucent and smooth 148 strains showed such small variations in colony morphology that they could be characterized as typical on both media Divergent results took the form of rough colonies in pure culture on both media in the case of two strains and mainly rough colonies with one strain The latter was separated into rough and smooth colonies but only the smooth form is included in the present material A few rough colonies on the agar medium only were found with two strains

Colonial pigmentation On Lowenstein Jensen medium 124 strains showed pale yellow colonies 28 were slightly paler and one had colonies of stronger yellow colour though not so strong that the strain could be regarded as scotochromogenic (4 5) *Short exposure to light* had no effect at all on the pigmentation The examinations were made in the majority of cases after three weeks growth at 37 Three dilutions were used so that the results of exposure to light could always be registered on test tubes containing few and well isolated colonies The cultures were then kept in daylight at room temperature until they were eight weeks old (*long exposure*) Reading at that time showed increased pigmentation in 46 cases

Drug resistance using Lowenstein Jensen medium to which the different drugs were incorporated was examined on 194 strains The results are shown in Table 3 (*limits of growth quantitatively similar* to that on control tube)

Distribution of the strains according to drug concentrations showed resistance in the majority of cases as compared to the highly sensitive culture of *M tuberculosis* (strain No 5) used as control That strain was inhibited by the lowest concentration of all drugs except viomycin

TABLE 3

M avium Animal Isolates

Drug Resistance

(Figures indicate distribution of 194 strains according to the highest concentration of drug (γ per ml) permitting quantitatively similar growth as on control tube Medium Lowenstein Jensen)

Drug	Concentration (code) 0	1	2	3	4	5
SM	0	0	47	146	1	--
PAS	0	0	0	7	187	
TSC	0	5	2	4	183	
INH	0	0	0	4	148	42
Viomycin	0	7	177	10	0	
Cycloserine	184	7	1	2	0	

Concentration of drug γ/ml

	Code 0	1	2	3	4	5
SM	0	2 0	4 0	16 0	64 0	
PAS	0	0 4	1 6	6 4	25 6	
TSC	0	0 4	1 6	6 4	25 6	
INH	0	0 08	0 32	1 28	8 0	50 0
Viomycin	0	10 0	40 0	160 0	640 0	
Cycloserine	0	32 0	64 0	128 0	256 0	

which inhibited growth at the next lowest concentration Only to cyclo serine were almost all the strains sensitive It will also be seen from the table that five strains gave growth only up to concentration 0 4 γ per ml thiosemicarbazone (TSC) One of these showed repeatedly this in creased sensitivity at concentrations 1 6 and 6 4 γ only at 25 6 γ the number of colonies was again similar to that on the control tube though the colonies were slightly smaller On account of the sensitivity to TSC these five strains were examined by all methods

Comparison with the limits for any growth (not given in the table) showed a displacement in the distribution corresponding to almost four times higher concentrations or one dilution step for streptomycin iso niazide and viomycin

Sensitivity to 100 units penicillin was examined on 153 strains Twenty one could be considered strongly resist nt since growth oc curred with the same number of colonies as on the control medium without penicillin 132 showed a smaller number of colonies but still some growth

The *effect of temperature on growth* on Lowenstein Jensen medium was examined on 58 strains The results ref r ing to the first time (in weeks) at which well isolated colonies wer visible are hown in the column diagrams in Fig 1 It will be seen that as regards growth at various temperatures the strains were absolutely uniform in the two main groups The majority grew though slowly at 22 At 37 and 40 all strains grew the majority in the course of two to three weeks At

in the rabbit experiments the strains isolated from cattle gave only slightly prolonged median survival times (3–4 days) as compared with those isolated from poultry and pigs No such displacement could be seen in the experiments on hens In consequence the source of the isolates was not taken into further consideration

Serotype Av II predominated among the serologically classified strains This will probably also apply to the other strains designated undetermined but the proportion is not known The results presented here are for two groups 1) the strains known to be Av II and 2) the undetermined strains comprising Av I spontaneously agglutinable and unclassified strains and those not tested serologically On account of the uniformity of the findings some of the results are given for both groups together

Colony morphology on Lowenstein Jensen and oleic acid albumin agar media was examined on 153 strains generally after three weeks using well isolated colonies Where growth on Lowenstein Jensen medium was typical the colonies were dysgonic and dome shaped with shiny smooth surface On agar medium the colonies were small and flat with somewhat irregular edge and undulated surface greyish white, quite translucent and smooth 148 strains showed such small variations in colony morphology that they could be characterized as typical on both media Divergent results took the form of rough colonies in pure culture on both media in the case of two strains and mainly rough colonies with one strain The latter was separated into rough and smooth colonies but only the smooth form is included in the present material A few rough colonies on the agar medium only were found with two strains

Colonial pigmentation On Lowenstein Jensen medium 124 strains showed pale yellow colonies 28 were slightly paler and one had colonies of stronger yellow colour though not so strong that the strain could be regarded as scotochromogenic (4 5) Short exposure to light had no effect at all on the pigmentation The examinations were made in the majority of cases after three weeks growth at 37 Three dilutions were used so that the results of exposure to light could always be registered on test tubes containing few and well isolated colonies The cultures were then kept in daylight at room temperature until they were eight weeks old (*long exposure*) Reading at that time showed increased pigmentation in 46 cases

Drug resistance using Lowenstein Jensen medium to which the different drugs were incorporated was examined on 194 strains The results are shown in Table 3 (limits of growth quantitatively similar to that on control tube)

Distribution of the strains according to drug concentrations showed resistance in the majority of cases as compared to the highly sensitive culture of *M tuberculosis* (strain No 5) used as control That strain was inhibited by the lowest concentration of all drugs except viomycin

TABLE 3

M avium *Animal Isolates*
Drug Resistance

(Figures indicate distribution of 194 strains according to the highest concentration of drug (γ per ml) permitting quantitatively similar growth as on control tube Medium Lowenstein-Jensen)

Drug	Concentration (code) 0	1	2	3	4	5
SM	0	0	47	146	1	
PAS	0	0	0	7	187	
TSC	0	5	2	4	183	
INH	*0*	*0*	*0*	*4*	*148*	*42*
Viomycin	0	7	177	10	0	
Cycloserine	184	7	1	2	0	

Concentration of drug γ/ml

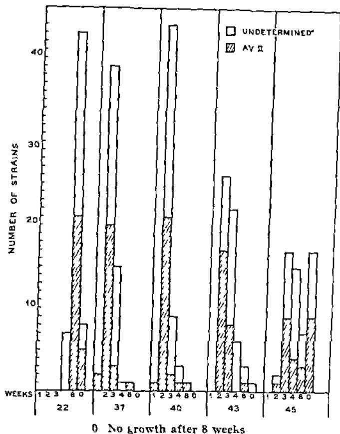
	Code 0	1	2	3	4	5
SM	0	2 0	4 0	16 0	64 0	
PAS	0	0 4	1 6	6 4	25 6	
TSC	0	0 4	1 6	6 4	25 6	
INH	0	0 08	0 32	1 28	8 0	50 0
Viomycin	0	10 0	40 0	160 0	640 0	
Cycloserine	0	32 0	64 0	128 0	256 0	

which inhibited growth at the next lowest concentration Only to cycloserine were almost all the strains sensitive It will also be seen from the table that five strains gave growth only up to concentration 0 4 γ per ml thiosemicarbazone (TSC) One of these showed repeatedly this increased sensitivity at concentrations 1 6 and 6 4 γ only at 25 6 γ the number of colonies was again similar to that on the control tube though the colonies were slightly smaller On account of the sensitivity to TSC these five strains were examined by all methods

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0 No growth after 8 weeks

Fig 1

M avium Animal isolates

Effect of temperature on growth on Lowenstein Jensen medium

43 there was growth of all strains except one but with a displacement towards slower growth At 45 this phenomenon was even more pronounced and many of the strains had not grown in the course of eight weeks

Comparison of the number of colonies after 8 weeks observation at the different temperatures showed almost identical germination at 37 and 40 There were fewer colonies in about 30 per cent of the strains at 22 in about 40 per cent at 43 and in about 80 per cent at 45

The *amidase test* was carried out with 152 strains the results are given in Table 4 All strains except one gave positive reaction to nicotinamide and pyrazinamide with an almost equal degree of positivity to the two amides Repeated tests with the negative strain (Av II) gave the same result The reactions to the other eight amides were all negative

The *sulphatase test* was carried out with 59 strains The results are given in Table 5 which shows both the time and degree of the first

TABLE 4

M avium *Animal Isolates*
Amidase Reactions

No of strains	Nicotinamide (Amide 5) 0	+	++	+++	Pyrazinamide (Amide 6) 0	+	++	+++
152	1	18	93	40	1	24	104	23

Reaction 0 = ≤ 2 γ + = > 2–5 γ ++ = > 5–10 γ +++ = > 10 γ

TABLE 5

M avium *Animal Isolates*
Sulphatase Test

Degree	First reaction 3 days	1 week	2 weeks	3 weeks	4 weeks	Total no of strains	Maximum reaction 3 days	1 week	2 weeks	3 weeks	4 weeks	Total no of strains
(+)	24	26	2	0	0	52	0	2	0	0	0	2
+	0	6	0	0	0	6	0	10	14	7	2	33
++	0	0	0	0	0	0	0	0	0	0	19	19
+++	0	0	0	0	0	0	0	0	0	1	3	4
Total	24	32	2	0	0	58	0	12	14	8	24	58

One strain completely negative after 4 weeks
Highest reaction +++ not ++++ as stated previously (3)

TABLE 6

M avium *Animal Isolates*
Catalase Test

Medium and Technique	Reaction 0	+	++	+++	++++	Total no of strains
Lowenstein Jensen Wayne	2	56	1	0	0	59
Lowenstein Jensen Ordinary	64	118	11	0	0	193
Lowenstein-Jensen + INH Ordinary	188	5	1	0	0	194

Highest concentration permitting growth [illegible] 100 colonies

positive reaction and similar observations for the maximum reaction It was found that most of the strains achieved a weak degree of positivity during the first week Only one strain was completely negative after four weeks This was not the strain that gave no reaction in the amidase test The distribution of the maximum reactions was spread over the period one to four weeks with the greatest number of reactions around + to ++

Table 6 shows the results of the *catalase test* using equal parts of

10 per cent Tween 80 and 30 per cent hydrogen peroxide The test was performed with the special technique described by Wayne (8) as well as on slants of Lowenstein Jensen medium and on the same medium containing the maximum concentration of INH permitting growth of ≥ 100 colonies It was found that the majority of the strains gave weak reactions or were negative with the largest number of negative reactions using the ordinary technique The degree of reaction was less on the medium containing INH 188 of the 194 strains being negative

The *niacin test* was carried out with 59 strains All the results were recorded as negative (*i e* ≤ 4 γ per ml)

The *Tween degradation test* was performed with 59 strains All results were negative when read after 7 days

The result of the *nitrate reduction test* was negative with all the 59 strains examined (*i e* ≤ 2 γ per ml)

Pathogenicity and Virulence

Hens (5 mg i v) The examinations were performed with 120 strains It will be seen from Table 7 that the distribution according to survival times is the same in the two main groups All the animals died the majority in less than 40 days The spleen was enlarged in most of the hens (101) Macroscopically visible processes were found in the liver of only few of the animals and only exceptionally in the lungs

TABLE 7
M avium *Animal Isolates*
Hens Survival Times (5 mg i v)

Serotype	Survival time in days									Total no of strains
	1–9	10–19	20–29	30–39	40–49	50–59	60–69	70–89	Killed	
Av II	0	1	16	10	5	1	0	0	0	33
Undetermined	0	6	29	37	10	4	1	0	0	87
Total	0	7	45	47	15	5	1	0	0	120

Table 8 shows the results of microscopy and culture Again there was no definite difference between the two main groups The spleens contained large numbers of bacteria also in the two cases where microscopy was negative The variability in microscopy findings was much greater in the lungs which must be taken as a sign of fewer bacteria in that organ even though culture gave maximal results in the majority of cases There was a conspicuously large number of negative microscopy results from the lungs in the undetermined group

Rabbits (5 mg i v) The examinations were carried out with 120 strains Table 9 shows the distribution according to survival times

TABLE 8

M avium Animal Isolates

Hens Findings by Microscopy and Culture (5 mg i v)

Serotype	Method	No of strains giving indicated results							
		Spleen				Lungs			
		0	1	2	3	0	1	2	3
Av II	Microscopy	0	0	1	32	1	6	17	9
	Culture	0	0	0	33	0	0	0	27
Undetermined	Microscopy	2	0	6	79	18	16	31	22
	Culture	0	0	0	84	0	0	0	74

Index	Microscopy	Culture
0	0	0
1	(+) +	+
2	++ +++	++
3	∞	+++ ∞

TABLE 9

M avium Animal Isolates

Rabbits Survival Times (5 mg i v)

Serotype	Survival time in days									Total no of strains
	1 9	10 19	20 29	30–39	40 49	50 59	60 69	70 89	Killed	
Av II	0	21	10	1	1	0	0	0	0	33
"Undetermined"	1	61	22	0	2	0	0	0	1	87
Total	1	82	32	1	3	0	0	0	1	120

from this aspect also there was uniformity in the two main groups Only one animal survived while all the others died the majority in less than 30 days

Macroscopical observation showed enlarged spleen in almost all of the animals (118) Processes in the joints were seen in about 15 per cent of the rabbits but only rarely in liver kidneys and lungs

It will be seen from Table 10 that there was no difference in the distribution of results of microscopy and culture between the two main groups The spleens contained large numbers of bacteria and the findings in the lungs particularly by microscopy showed great variability thus indicating the presence of fewer bacteria in that organ The variation in results and the few maximal values found by culture from the joints were doubtless due to the short survival times

Guinea pigs (1 mg i v) 120 strains were examined each injected into two animals the first injected called guinea pig 1 and the second guinea pig 2 Since the distributions of survival times for these two

TABLE 10

M avium *Animal Isolates*
Rabbits Findings by Microscopy and Culture (5 mg i v)

Serotype	Method	No of strains giving indicated results											
		Spleen				Lungs				Joints			
		0	1	2	3	0	1	2	3	0	1	2	3
Av II	Microscopy	0	0	1	32	3	6	15	9				
	Culture	1	0	0	32	0	0	2	29	0	21	10	2
Undetermined	Microscopy	0	2§	1	83	14	23§	40	9				-
	Culture	0	0	0	86§	0	1	3	83§	6	52	25	4§

For Index see Table 8

Microscopy ∞
§ One rabbit survived the observation period
Not examined

TABLE 11

M avium *Animal Isolates*
Guinea Pigs 1 and 2 Survival Times (1 mg i v)

Serotype	Survival time in days						Killed	Total no of guinea pigs
	1–9	10–19	20–29	30–39	40–49	50 59		
Av II	1	1	7	15	11	10	21	66
Undetermined	1	1	30	36	45	21	40	174
Total	2	2	37	51	6	31	61	240

TABLE 12

M avium *Animal Isolates*
Guinea Pigs 1 and 2 Results of Microscopy of Smears from Organs (1 mg i v)

Organ	Microscopy						Total no of guinea pigs
	0	(+)	+	++	+++	∞	
Spleen	40	7	15	47	47	83	239
Lungs	145	33	41	17	2	0	238

groups were the same they are shown together for all 240 animals in Table 11 It will be seen that four animals died within 20 days and that only 61 survived the whole observation period i e 25 per cent of the infected animals The survival times for the remaining animals were fairly evenly distributed over the period 20 to 59 days The spleen was generally enlarged in one or both of the animals and only occasionally were there macroscopically visible processes in the lungs

TABLE 13

M. avium Animal Isolates

Guinea Pigs 1 and 2 Results of Culture from Organs (1 mg i.v.)

Organ	Culture 0	+	++	+++	∞	Cont	Total no of guinea pigs
Spleen	0	13	8	12	205	2	240
Lungs	0	22	24	64	129	1	240

Cont = contaminated

The results of microscopy and culture from the spleen and lungs were also analysed separately for all the guinea pigs in the two groups. Here again the distributions were so alike that the results could be taken together. It will be seen from Tables 12 and 13 that there were more bacteria in the spleen than in the lungs and that this difference is more evident by microscopy than by culture.

TABLE 14

M. avium Animal Isolates

Guinea Pigs 1 and 2 Comparison of Bacterial Index (Spleen) for Guinea Pigs Infected with the Same Strain (1 mg i.v.)

Guinea pig 2	Guinea pig 1: 0	1	2	3	4	5	Total no of strains
0	0	0	0	0	0	0	0
1	0	5	2	4	0	1	12
2	0	1	2	3	2	2	10
3	0	2	3	13	3	1	22
4	0	1	1	12	5	13	32
5	0	2	6	5	4	24	41
Total	0	11	14	37	14	41	117

Index	Microscopy	Culture
0	0	0
1	0	+ ++ +++
2	{ 0 { (+)	{ ∞ { +++ ∞
3	+ ++	All ∞
4	+++	All ∞
5	∞	All ∞

The results of microscopy and culture are given in the form of a bacterial index. On account of the superiority of culture to microscopy the bacteria corresponding to ≤ ++ by culture could not be demonstrated by microscopy (with occasional exceptions). Therefore the results by the two methods are supplementary to each other by graduat

ing the low and high numbers of bacteria. This relation between the two methods forms the basis of the index covering values from 0 to 5 (for details see Table 14). Comparison of the indices for the two guinea pigs infected with the same strain showed great differences and there did not appear to be any relation between the results. This can be seen clearly in the case of the spleen examinations (Table 14).

TABLE 15

M. avium. Animal Isolates
Distribution of 120 Strains According to Survival or Death of Two Guinea Pigs Infected with the Same Strain (1 mg i.v.)

Guinea pig 2	Guinea pig 1 Survival	Guinea pig 1 Spontaneous death	Total no. of strains
Survival	10 (7.8)	22 (24.3)	32
Spontaneous death	19 (21.2)	69 (66.7)	88
Total no. of strains	29	91	120

Figures in brackets represent expected values due to random distribution.

TABLE 16 a

M. avium. Animal Isolates
Guinea Pigs (1mg i.v.). Distribution According to Average Bacterial Index for Spleen Within the Groups of Strains Where both Animals Survived or Were Killed by the Infection

Survivors	Average bacterial index – spleen 0.0	0.5	1.0	1.5	2.0	2.5	3.0	3.5	4.0	4.5	5.0	Total no. of strains
2	0	0	5	1	4	0	0	0	0	0	0	10
0	0	0	0	0	0	0	10	17	7	11	22	67

TABLE 16 b

Distribution According to Bacterial Index for Spleen Within the Groups of Strains Where only One Animal Survived the Infection

Guinea pig	Fate	Bacterial index – spleen 0.0	1.0	2.0	3.0	4.0	5.0	Total no. of strains
1	s	0	5	4	7	1	2	19
2	s	0	4	5	4	7	1	21
1	d	0	0	3	7	3	8	21
2	d.	0	0	2	6	4	7	19

s = survived the observation period   d = died spontaneously
For Index see Table 14

The distribution of the 120 strains according to survival or death of the two guinea pigs is shown in Table 15. 179 out of 240 guinea pigs

died (75 per cent) The figures in brackets indicate the expected values due to random distribution There seemed to be only slight preponderance of equal results for the two animals this weakly positive correlation was not significant

However examination of the bacterial index and the fate of the animals showed a clear relationship between content of bacteria in the organs and survival or death For the strains where both guinea pigs died or survived the infection there was a better correlation between the bacterial index for the two animals It was therefore considered reasonable to calculate an average index Tables 16 a and 16 b show the distribution of these averages for the spleen When both animals survived the index was low and the distribution quite different from that observed when both guinea pigs died Where only the one animal died (Table 16 b) the findings lay between the two other groups with a *considerably higher index for the animals that died though not quite* as high a value as in the group where both guinea pigs died

Guinea pigs (1 mg i p) 120 strains were examined each injected into two animals Fifteen of these died spontaneously spread over the whole observation period Only in one instance did both animals die (28th and 31st days) Since five out of the six cultures were contaminated and the remaining culture (spleen) gave growth of only few bacteria the two animals probably died of intercurrent disease

Macroscopically visible local processes in the omentum were very frequent only in 13 cases could they not be demonstrated

Since few animals died and microscopy of the organs showed bacteria in only a minority of the animals the content of bacteria in the organs of these guinea pigs is illustrated only by an average bacterial index based on the results of culture (Table 17) It will be seen that the spleen contained more bacteria than the lungs and that only few of the animals showed maximal index values

TABLE 17

M avium *Animal Isolates*

Guinea Pigs Distribution of Strains According to Average Bacterial Index (Culture Only) (1 mg i p)

Organ	Average bacterial index (Culture) 0 0	0 5	1 0	1 5	2 0	Total no of strains
Spleen	0	6	103	7	0	116
Lungs	10	40	64	4	0	118

Index	Culture
0	0
1	+ ++ +++
2	∞

TABLE 18

M. avium Animal Isolates

Mice. Distribution of 59 Strains According to Number of Survivors (10 mg i.p.)

No. of surviving mice	Serotype Av II a	b	c	Undetermined a	b	c
0	1	0	4	0	0	0
1	0	0	3	0	0	4
2	2	0	4	0	0	1
3	0	0	4	1	0	8
4	7	1	5	5	1	10
5	7	9	5	9	3	7
6	10	17	2	17	28	2

a = House mice b = White mice c = Red mice

Mice (10 mg i.v.). 59 strains were examined, each injected on six mice per mouse breed. Spontaneous deaths occurred with varying frequency in the different breeds. Table 18 shows the distribution of the strains with regard to the number of survivors at the end of the observation period. Spontaneous deaths occurred to a different extent among the three breeds of mice (16, 57 and 157 animals respectively). There was no definite difference in the distribution of these deaths within the two main groups. The deaths among the white mice were spread evenly over the whole observation period. As regards the house and red mice, there was an accumulation of deaths during the first ten days.

TABLE 19

M. avium Animal Isolates

Mice. Distribution of Strains According to Average Bacterial Index (Microscopy) Calculated per Mouse per Organ (10 mg i.p.)

Organ	Average index	Serotype Av II a	b	c	Undetermined a	b	c
Spleen	0	0	0	0	0	0	0
	0.1 - 1.4	0	0	[illegible]	0	0	5
	1.5 - 2.4	2	1	5	1	0	13
	2.5 - 3.0	24	26	17	31	32	13
Lungs	0	0	0	2	0	0	1
	0.1 - 1.4	0	0	5	2	1	15
	1.5 - 2.4	9	15	9	17	15	8
	2.5 - 3.0	17	12	7	13	16	7

Only the mice that survived the two months observation period are included in the evaluation.

a = House mice b = White mice c = Red mice

Index (microscopy):

0 = 0 bacteria 2 = 51–300 bacteria

1 = 1–50 bacteria. 3 = ? per sight field to ∞ bacteria

TABLE 30

Typical Pattern of Danish M avium Strains and Number of Divergencies

(≤ 5 per cent)

In vitro *Experiments*

Feature		Typical range	No of divergencies	Feature		Typical range	No of divergencies
Examination of colonies 153 strains							
Morphology LJ		See text	3	Pigmentation LJ		Pale yellow	1
Morphology O A		See text	5	Light short exposure		Unchanged	0
				Light long exposure		Variable	
Drug resistance 194 strains							
SM		4 – 16 γ	1	INH		8 – 50 γ	4
PAS		64 – 256 γ	0	Viomycin		10 – 160 γ	0
TSC		64 – 56 γ	7	Cycloserine		≤ 32 γ	3
Effect of temperature on growth 58 strains							
Growth at	25	≥ 4 wks to 0 8 wks	0	No of colonies compared with 37	25	≤	0
	37	2 to 4 wks	3		40	≤	0
	40	2 to 4 wks	[illegible]		43	≤	0
	43	[illegible] to 8 wks	1		45	≤	0
	45	≥ 3 wks to 0 8 wks	2				
Amidase test 15? strains							
Amide		+ to +++	1	Other 8 amides		0	0
Amide 6		+ to +++	1				
Sulphatase test 59 strains							
Fi t positive reaction	Time	3 days to 1 week	2	Maximum positive reaction	Time	1 to 4 weeks	0
	Degree	(+) to +	1		Degree	+ to +++	3
Catalase test 59 193§ 194† strains							
Wayne		+	3	Ordinary§		0 to ++	0
				INH ordinary†		0	6
Niacin test (a) Tween degradation (b) Nitrate reduction (c) 59 strains							
(a) (b) (c)		0	0				

... Strains and Number of Divergencies
(≤ 5 per cent) In vivo experiments

Feature		Typical range	No of divergencies	Feature		Typical range	No of divergencies
Hens 5 mg i v 120 strains							
Survival time		10 – 59 days	1	Micr	Spleen	+++ to ∞	2
Macr	Spleen	Variable			Lungs	Variable	
	Lungs	0	2	Culture	Spleen	∞	0
					Lungs	+++ to ∞	0
Rabbits 5 mg i v 120 strains							
Survival time		10 – 39 days	5	Micr	Spleen	++ to ∞	2
Macr	Spleen	Enlarged	2		Lungs	Variable	
	Kidneys	0	3	Culture	Spleen	∞	1
	Lungs	0	4		Lungs	+++ to ∞	6
	Joints	Variable			Joints	+ to ∞	6
Guinea pigs 1 mg i v 120 strains							
Macr	Spleen	Enlarged in one or both g ps	0	Survival time and average *index* (micr and culture of spleen)		(a) One *or* both g ps die	
	Lungs	No processes in one or both g ps	0			(b) If both g ps survive index > 1	5
Guinea pigs 1 mg i p 120 *strains*							
Local processes		+ in one or both g ps	2	Average index (culture of spleen)		> 0 5	6
House mice 10 mg i p 59 strains							
Survivors (of 6)		3 – 6	3	Average index (micr)	Spleen	3	3
Average index	Proc in organs	< 0 5	0		Lungs	2 – 3	2
White mice 10 mg i p 59 strains							
Survivors (of 6)		4 – 6	0	Average index (micr)	Spleen	3	1
Average index	Proc in organs	< 0 5	0		Lungs	2 – 3	1
Red mice 10 mg i p 59 strains							
Survivors (of 6)		Variable		Average index (micr)	Spleen	1 – 3	0
Average index	Proc in organs	< 0 5	0		Lungs	1 – 3	3

Autopsy after two months showed very few macroscopically visible processes in the organs The numbers of bacteria in spleen and lungs determined by microscopy are shown in Table 19 Only mice that survived the whole observation period are included in the evaluation The strains that killed all the mice are therefore not stated in the table

It will be seen from the distribution of the strains according to average index that the surviving house and white mice reacted fairly uniformly to the infection The results of microscopy of the spleen were almost constant at the maximum value while the results from the lungs were more variable Red mice had on the whole fewer bacteria in the lungs

TYPICAL PATTERN OF *M AVIUM*

The limits for *typical* results by the various methods were determined as regards the quantitative evaluations in such a way that at least 95 per cent of the strains were within that range The procedure was justifiable since none of the distributions showed more than one mode Correspondingly with the qualitative evaluations the results were considered typical if they were achieved with about 95 per cent of the strains Since there was only little missing information (including unsuccessful examinations) the error in including such results among the typical is of no practical importance

A feature was considered *variable* if all the possibilities were represented in at least 5 per cent of the strains

Divergent results were those lying outside the stated limits They are not registered for features regarded as variable

Tables 20 (*in vitro*) and 21 (*in vivo*) show the individual features together with the limits for the typical results and the number of divergencies for the total number of strains included in the various experiments

It will be seen that the total number of features examined was 74 40 in the *in vitro* and 34 in the *in vivo* experiments Of these 1 and 5 respectively are regarded as variable

The number of divergencies with the 59 strains examined by all the methods was 73 34 from the *in vitro* and 39 from the *in vivo* experiments Distribution of these divergencies over the individual strains showed that 23 strains had from one to two 11 strains from three to four and only one strain six divergencies This distribution was independent of the kind of animal from which the strain originated and whether or not the strains were serotyped The Av I strain showed only one divergency

(For Discussion see Part 2 of this study (2))

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MYCOBACTERIUM AVIUM

A Bacteriological and Epidemiological Study of M avium Isolated from Animals and Man in Denmark

Part 2 Strains Isolated from Man

By

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MATERIAL AND METHODS

As mentioned previously (16) the strains isolated from man included in this study were limited to those which could be classified as *M avium* There were 22 strains (Table 1) all of which were serotyped 16 by *Schaefer* (42) and 6 by *Bennedsen* (4) Part of the material was reported in previous publications (see Table 2) though before it was possible to serotype the strains The table shows the SSC (Statens Seruminstitut Collection) numbers and references to the previous papers

It will be seen from Table 1 that 17 strains were isolated from specimens from the respiratory system and 5 from extrapulmonary processes The four strains of no clinical significance were isolated only once and with only few colonies One person was completely healthy but had participated in routine group tuberculosis examination (Av I) One patient had pleural metastasis following hypernephroma post mortem examination revealed that the lungs were normal (Av III) One patient had cor pulmonale (Davis) The strains were regarded as clinically significant when they were isolated repeatedly (generally with many bacteria) from patients with pulmonary processes including silicotic lesions chronic bronchitis and bronchiectasis or when the patient died before further examinations could be performed The extrapulmonary processes were located in the glands in three cases (one Av II two Watson) one in tendon sheaths (Av II) and one in bones (Davis)

It will also be seen from Table 1 that Av II and Davis serotypes formed the main part of the material with 9 and 7 strains respectively Av I was represented with one strain and the other serotypes with only one or two These latter have been assembled in one group called other types in some of the subsequent tables Av I is not included in that group since a strain of similar serotype in Part 1 of this study could not be distinguished from Av II

The methods used were as described in Part 1 of this study (16)

RESULTS

Colony morphology on Lowenstein Jensen and oleic acid albumin agar media was typical with all strains

Colonial pigmentation On Lowenstein Jensen medium the colonies were pale yellow only one strain (Av II) gave growth of slightly paler colonies *Short exposure to light* had no effect on the pigmentation while *long exposure* increased the colour in 15 cases (1 Av I 3 Av II 7 Davis 4 "other types") The seven strains which were not affected were 6 Av II and 1 other types

TABLE 1

M avium *Human Isolates*

No of Strains Divided According to Localization of Disease and Clinical Significance for Person Concerned

Serotype	No of strains			
	Pulmonary		Extrapulmonary	Total
	Significant	Non significant	Significant	
Av I	0	1	0	1
Av II	6	1	2	9
Davis	5	1	1	7
Av III	0	1	0	1
Av IV	1	0	0	1
Watson	0	0	2	2
New type	1	0	0	1
Total	13	4	5	22

TABLE 2

M avium *Human Isolates*

SSC (Statens Seruminstitut Collection) Numbers and References to Previous Publications

Serotype	No of strains	SSC No	Reported in previous paper as M *avium*
Av I	1	326	11 (erroneously stated as SSC 321)
Av II	9	314	11 14
		318	11
		319	11 15 (*case 2*)
		320	11 12 (pt no 18)
		779	14 46
		794	—
		580	15 (case 4) 14
		602	15 (case 3) 14
		1101	—
Davis	7	222	—
		328	13
		330	13
		619	15 (case 7) 14 46
		784	—
		1007	—
		1102	—
Av III	1	89	—
Av IV	1	315	11 14
Watson	2	327	12 (pt no 22)
		999	—
New type	1	317	11 13

TABLE 3

M avium *Human Isolates*
Drug Resistance

(Figures indicate the distribution of ?2 strains according to the highest concentration in γ per ml of drug permitting quantitatively similar growth as in control tube Medium Lowenstein-Jensen)

Drug	Concentration (code) 0	1	2	3	4	5
SM	0	0	4	11	7	
PAS	0	0	1	0	21	
TSC	0	2	1	0	19	
INH	0	0	0	9	4	9
Viomycin	0	0	13	8	1	
Cycloserine	?1	1	0	0	0	

Concentration of drug γ per ml

	Code 0	1	2	3	4	5
SM	0	?0	4 0	16 0	64 0	
PAS	0	0 4	1 6	6 4	25 6	
TSC	0	0 4	1 6	6 4	25 6	
INH	0	0 08	0 3?	1 28	8 0	50 0
Viomycin	0	10 0	40 0	160 0	640 0	
Cycloserine	0	32 0	64 0	128 0	256 0	

The results of *drug resistance determination* on Lowenstein Jensen medium are shown in Table 3 where the whole material is taken together The distribution of the strains in this total material was different in some respects from that of the M *avium* strains in Part 1 Table 3 These differences are as follows

SM 7 strains under code 4 (64 γ) was a particularly large number (1 Av I 1 Av II 3 Davis and 2 other types)

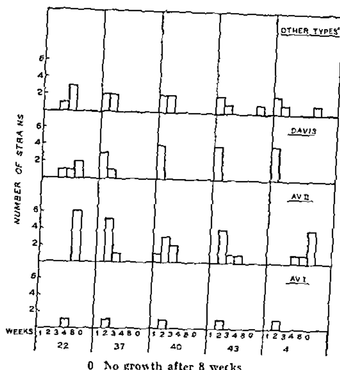
PAS The one strain under code 2 (1 6 γ) was Davis

TSC This distribution was in itself remarkable with 2 strains under code 1 As both these strains were Davis it was thus shown that as with M *avium* in Part 1 this serotype also included some TSC sensitive strains

INH The 9 strains under code 3 (1 28 γ) was also a remarkably large number (7 Davis 2 other types)

100 units penicillin 9 strains were considered strongly resistant as they gave growth of the same number of colonies as on the control medium without penicillin (6 Davis 3 other types) 12 strains had a lesser number of colonies but gave some growth (1 Av I 9 Av II 1 Davis 1 other types) The New type strain was inhibited completely and was thus the only strain that was strongly sensitive to the concentration of penicillin used

Conclusion As compared with Av II the Davis strains showed a tendency towards a higher degree of resistance to SM and penicillin and a lesser degree to INH



0 No growth after 8 weeks

Fig 1

M avium Human isolates
Effect of temperature on growth on Lowenstein Jensen medium

The *effect of temperature* on growth on Lowenstein Jensen medium was examined on the following number of strains 1 Av I 6 Av II 4 Davis 4 other types (one of each serotype) The results referring to the first time (in weeks) at which well isolated colonies were visible will be seen from Fig 1 There was a characteristic difference in the distributions between Av II and Davis at all temperatures Davis showed earlier growth and at 40 43 and 45 all the strains gave growth in the course of one week At 45 the difference was most pronounced Four of the Av II strains showed no growth in the course of eight weeks Of the other types the New type strain was distinct in that no growth could be registered in the course of eight weeks either at 43 or at 45°

As regards the number of colonies after eight weeks observation at the different temperatures both the Av II strains which grew at 45 had fewer colonies at that temperature than at 37 Only one of the eight other strains that grew at 45 showed similar growth

Conclusion Temperature experiments (from 22 to 45) showed that strains of serotype Davis grew more rapidly than Av II strains 45 provided the sharpest distinction between the strains The new type strain was different from the others at 43

Amidase test (Table 4) All strains were positive to amides 5 and 6 and the degree of positivity was distributed fairly uniformly with the two amides independent of serotype There was no reaction to any of the other eight amides

Sulphatase test (Table 5) The majority of the strains gave a weak degree of positive reaction in the course of two weeks and there was no systematic difference between the various serotypes either with regard to the time or degree of first registrable and maximum reactions

TABLE 4
M avium *Human Isolates*
Amidase Reactions

No of strains	Nicotinamide (Amide 5)				Pyrazinamide (Amide 6)			
	0	+	++	+++	0	+	++	+++
22	0	4	13	5	0	5	12	5
Reaction	0 = ≤ 2 γ	+ = > 2-5 γ	++ = > 5-10γ	+++ = > 10 γ				

TABLE 5
M avium *Human Isolates*
Sulphatase Test

Degree	First reaction						Maximum reaction					
	3 days	1 week	2 weeks	3 weeks	4 weeks	Total no of strains	3 days	1 week	2 weeks	3 weeks	4 weeks	Total no of strains
(+)	5	8	4	1	0	18	0	0	0	0	0	0
+	2	0	1	1	0	4	0	1	3	4	2	10
++	0	0	0	0	0	0	0	0	0	1	5	6
+++	0	0	0	0	0	0	0	0	1	1	4	6
Total	7	8	5	2	0	22	0	1	4	6	11	22

TABLE 6
M avium *Human Isolates*
Catalase Test

Medium and technique	Reaction					Total no of strains
	0	+	++	+++	++++	
Lowenstein-Jensen Wayne	0	9	9	1	0	19
Löwenstein-Jensen Ordinary	4	6	3	0		15
Lowenstein-Jensen + INH Ordinary	11	3	0	1	0	15

Highest concentration permitting growth of ≥ 100 colonies

The results of the *catalase test* are shown in Table 6 With the special technique (*Wayne* (44)) there was a remarkably large number of strains with $\geq$ + + reaction Of the ten strains 1 was Av I 6 were Davis and 3 were other types With the ordinary technique 4 Davis and 1 Av III gave reactions $\geq$ + + On medium containing INH reactions $\geq$ + were found only with Davis strains

The *niacin, Tween degradation* and *nitrate reduction tests* were all negative except for one Davis strain that gave a weakly positive reaction in the niacin test

Conclusion Serotype Davis and other types generally gave stronger catalase reaction than Av II

Pathogenicity and Virulence

Hens (5 mg i v) It will be seen from Table 7 that there was no difference between the various serotypes as regards the distribution of survival times except for two other types strains that were not able to kill the hens in the course of the observation period (Av IV New type) All the other animals died in less than 50 days

TABLE 7
M avium *Human Isolates*
Hens Survival Times (5 mg i v)

Serotype	Survival time in days 1–9	10–19	20–29	30–39	40–49	50–59	60–69	70–89	Killed	Total no of strain
Av I	0	0	0	1	0	0	0	0	0	1
Av II	0	0	3	4	2	0	0	0	0	9
Davis	0	1	0	5	1	0	0	0	0	7
Other types	0	0	0	1	2	0	0	0	2	5
Total	0	1	3	11	5	0	0	0	2	22

Microscopy (Table 8) was not carried out systematically and the results are thus difficult to evaluate However they indicate more bacteria with Av II than with Davis and other types strains

By culture from the spleen the maximum number of colonies could be recovered with all strains except one (New type) but there were slightly less colonies from the lungs with Davis strains than with Av II

Rabbits (5 mg i v) Table 9 shows that there was no difference in the distribution of survival times between the various serotypes except for two strains (Davis New type) where the animals survived the observation period All the rest died in less than 40 days This Davis strain killed the hen in 37 days

Microscopy (Table 10) was not carried out systematically but the

TABLE 8

M avium Human Isolates

Hens Findings by Microscopy and Culture (5 mg i v)

Serotype	Method	No of strains giving indicated results — Spleen 0	1	2	3	Lungs 0	1	2	3
Av I	Microscopy	0	0	0	1	0	0	1	0
	Culture	0	0	0	1	0	0	0	1
Av II	Microscopy	0	0	1	1	0	0	1	0
	Culture	0	0	0	9	0	0	0	7
Davis	Microscopy	0	1	1	1	3	0	0	0
	Culture	0	0	0	7	0	1	2	4
Other types	Microscopy	1	1	0	0	1	1	0	0
	Culture	1	0	0	3	1	0	1	2

Index	Microscopy	Culture
0	0	0
1	(+) +	+
2	++ +++	++
3	∞	+++ ∞

TABLE 9

M avium Human Isolates

Rabbits Survival Times (5 mg i v)

Serotype	Survival time in days 1–9	10 19	20 29	30 39	40 49	50 59	60–69	70 89	Killed	Total no of strains
Av I	0	1	0	0	0	0	0	0	0	1
Av II	0	6	3	0	0	0	0	0	0	9
Davis	0	5	0	1	0	0	0	0	1	7
Other types"	0	1	2	1	0	0	0	0	1	5
Total	0	13	5	2	0	0	0	0	2	22

results indicate more bacteria with Av II than with Davis and other types strains Table 10 also shows that by culture maximum values were obtained from the spleen in the majority of cases However 2 Davis and 2 other types (Av III New type) gave fewer colonies and there were slightly less colonies from the lungs with Davis strains than with Av II

It should be mentioned that experiments carried out at intervals of some years with three strains (Davis Av IV New type) gave variable results as regards virulence for both rabbits and hens

Guinea pigs (1 mg i v) The experiment comprised the following strains 1 Av I 7 Av II 7 Davis and 5 other types It will be seen

was no definite difference in the frequency of deaths caused by Av II and Davis Autopsy after two months showed very few macroscopically visible processes in the organs The number of bacteria in the organs determined by microscopy (Table 14) showed no marked difference in the results for the three mouse breeds Comparison between Av II and Davis as groups showed definitely less bacteria in the organs after infection with Davis strains The results with other types strains were about the same as with Davis

TABLE 13

M avium Human Isolates

Mice Distribution of Strains According to Number of Survivors

No of surviving mice	Serotype								
	Av II			Davis			Other types		
	a	b	c	a	b	c	a	b	c
0	0	0	0	0	0	0	0	0	0
1	0	0	1	0	0	0	0	0	0
2	0	0	1	0	0	1	0	0	0
3	0	0	2	1	0	1	0	0	1
4	1	0	1	2	0	2	0	0	0
5	0	1	0	1	0	1	0	0	2
6	4	4	0	3	7	2	3	3	0

Mouse experiments not performed with Av I strain

a = House mice   b = White mice   c = Red mice

TABLE 14

M avium Human Isolates

Mice Distribution of Strains According to Average Bacterial Index (Microscopy) Calculated per Mouse per Organ (see text)

Organ	Average index	Serotype								
		Av II			Davis			Other types		
		a	b	c	a	b	c	a	b	c
Spleen	0	0	0	0	1	0	2	0	1	0
	0 1 – 1 4	0	0	1	2	3	4	1	0	2
	1 5 – 2 4	0	1	1	2	4	1	0	1	1
	2 5 – 3 0	5	4	3	2	0	0	2	1	0
Lungs	0	0	0	0	2	2	4	1	1	1
	0 1 – 1 4	1	1	2	4	2	3	2	1	2
	1 5 – 2 4	1	3	1	1	3	0	0	1	0
	2 5 – 3 0	3	1	2	0	0	0	0	0	0

Only the mice that survived the two months observation period are included in the evaluation

a = House mice   b = White mice   c = Red mice

Index (microscopy)

0 = 0 bacteria
1 = 1–50 bacteria
2 = 51–300 bacteria
3 = 2 per sight field to ∞ bacteria

Comparison with the Typical Pattern of M avium Strains from Animal Sources in Part 1

Fifteen of the strains isolated from man were examined by the same number of methods as were included in the description of the typical pattern of the animal strains These were 5 Av II 7 Davis 1 Av III 1 Watson and 1 New type The number of divergencies from the typical pattern was counted for each of these strains according to the same principles as in Part 1

TABLE 15
M avium Human Isolates
Distribution of Strains According to Divergencies from the Typical Pattern of M avium II

Serotype	No of strains	No of divergencies 0	1–2	3–4	5–9	10–19	20–29
Av II	5	1	3	0	1	0	0
Davis	7	0	0	0	0	7	0
Av III	1	0	0	0	0	1	0
Watson	1	0	0	0	0	1	0
New type	1	0	0	0	0	0	1

The results of this comparison are shown in Table 15 It will be seen that Av II strains are not distinguishable from the strains isolated from animals Three strains had from one to two divergencies and only one had five The Davis strains Av III and Watson all had more than ten divergencies and New type the largest number viz 22 The divergent results are fairly evenly distributed between the *in vivo* and *in vitro* experiments in the latter mainly under the temperature experiments and the catalase test and to a lesser degree in the resistance determinations Only the New type strain had the majority of its divergencies (18 out of 22) in the *in vivo* experiments

DISCUSSION

The present work shows that manifest infections with mycobacteria in animals in Denmark are almost always caused by *M avium* virulent for hens and rabbits (for description of *M avium* see *Feldman* (18)) Only in two cases were other mycobacterial strains isolated The one from a heifer was classified by *Schaefer* (42) as serotype Yandle Whether or not the other strain (Unclass special) is of pathogenic significance cannot be determined since it was isolated from a specimen of soil from a poultry run

The material comprises a large number of [illegible] and the results for each feature were uniform independent of [illegible] the strains were Av II or undetermined Since none of [illegible] tions showed more

than one mode the limits for the typical results for *M avium* for each feature were determined in such a way that the range covered about 95 per cent of the strains Comparison of the individual strains with the typical pattern is made as a census of the number of divergencies In this way each feature is afforded equal weight even though all features are not independent of each other, e g the effect of temperature on growth In the virulence examinations also there is a relationship between the survival time the processes in the organs and the number of bacteria No consideration has been given in this work as to whether it is reasonable to include all the 74 features examined in the evaluation of the strains

The 73 divergencies were distributed fairly evenly among the strains regardless of whether or not the strains were serotyped one to two divergencies being found with 23 strains and three to four with 11 strains The large number of divergencies with one strain (six) is due to the dependence of the features on each other (rabbit with long survival time and consequent processes in organs) Since serotyping of a representative part of the material showed that Av II predominated this will probably also apply to all the strains The typical pattern of *M avium* based on all the results in the first part of the study will therefore presumably also describe serotype Av II From the distribution of the divergencies it can thus be predicted that a strain which shows more than six features diverging from the typical pattern will be found to be of a serotype other than Av II (or Av I)

This conclusion is not restricted by the special selection of the material since strains eliminated by the primary screening have a considerably larger number of divergencies than six e g *M bovis* *M xenopei*

This material provides no information concerning the dependence of the divergencies on variations in technique and experimental animals Repeated experiments would be necessary to ascertain this For the same reason it is not possible to determine whether the different distributions in bacterial index for guinea pigs infected intravenously have any connection with special characteristics of the strain The divergent results in the resistance determinations to TSC and in the amidase test have been confirmed by repeated examinations thus showing that they are connected with the strains Strains with relative sensitivity to TSC were not found by *Marks & Richards* (27) *Marks & Trollope* (28) *Collins* (10) and *Kubin et al* (24) who used a highest concentration of 10 γ per ml and found all strains to be strongly resistant However *Meissner* (34) found a few strains with partial resistance to 1 γ per ml

The result of the amidase test was extraordinary with one strain there being no positive reaction to nicotinamide (amide 5) and pyrazinamide (amide 6) According to *Bonicke* (7 8) and to our own experience positive reaction to these amides is typical for *M avium*

Only a few other results with *M avium* will be discussed here

The optimal growth of *M avium* at 37–40 is in agreement with *Feldman* (18) and *Bergey s Manual* (5) where it is stated that growth occurs at 39–40 and 40 respectively The temperature range in these works is given as 25–45 and 30–44 The present results with *M avium* at 45 are in best concordance with *Marks & Richards* (27) who report that only one third of the strains gave growth at that temperature in the course of three weeks As stated by *Runyon* (39) *Kovacs* (21) and *Marks & Trollope* (29) growth at 45 suggests *M avium*

Since *M avium* grows slowly at 22 the length of the observation period is of significance in comparing the results Taking this factor into consideration there is good correlation between our results at 22 and those reported by *Kovacs* (21) and *Meissner* (31 30 32 33) after an observation period of 3–4 weeks

Concerning the biochemical tests the weak catalase reaction is not in agreement with *Feldman* (19) and our finding of weakly positive reactions in the sulphatase test does not confirm the results of *Kubica & Beam* (23) nor those of *Tarshis* (43) *Runyon* found a distinction "but it was a quantitative difference and thought to be not sufficiently dependable to use routinely (39)

The human isolates comprise only strains classified as *M avium* *Even though three of these (Davis Av IV New type) showed different* degrees of virulence in repeated experiments at intervals of several years the results obtained with them justify the diagnosis The only strain that caused some difficulties was that classified as New type (13)

The special selection of strains in this study must be borne in mind when comparing with works dealing with avian like strains which by definition include only strains attenuated for hens (2)

The fact that the material included seven different serotypes dominated by Av II and Davis (42 4) provided the possibility for a new evaluation of the results by comparison with the typical pattern of *M avium*

Fifteen of the human isolates were examined to such an extent that they could be compared individually with the pattern described in Part 1 This comparison showed a characteristic relationship between serotype and number of divergencies The Av II strains from humans resembled the typical pattern and the Av I showed only few divergencies (The examinations with the latter strain were not complete) The other strains however were clearly different from Av II with more than ten divergencies

The material of Davis strains is fairly rep[illegible] but it is so small that it provides only a limited descri[illegible]on of th[illegible] Da[illegible]is strains as a group

It will be seen from the tables that [illegible] *in* [illegible]d *in vivo* the Davis strains gave results that deviated from [illegible] in similar examina

tions with Av II strains If these differences lie within the selected limits they do not reveal themselves by the number of divergencies (e g resistance to viomycin) In other instances where part of the distribution lies outside the limits it becomes fortuitous with which of the strains divergencies are observed This reduces the number of divergencies with the various strains but seems to give a more realistic evaluation of the results

In comparing Av II and Davis it is of special interest that with the doses used here the Davis strains were generally only slightly less virulent than Av II for the classical experimental animals (hens and rabbits) The infections caused a quite different pattern from that obtained with similar doses of a number of Battey strains from U S A examined here (11) These strains have not yet been serotyped Furthermore the distributions according to survival times in the present work show shorter survival times for rabbits than for hens infected with the same dose This applies both to *M avium* and Davis strains The contrast between the marked resistance of rabbits and the various degrees of susceptibility in chickens to Group III mycobacteria (infection dose 10^{-3} mg) reported by *Feldman & Ritts* (20) has thus not been demonstrated with Davis strains in the present work However such a contrast has been found to be characteristic for *M xenopei* (17) The quite large dose of 5 mg was chosen because a number of experiments with attenuated strains had shown that that dose provoked chronic processes in joints and tendon sheaths of rabbits within the observation period of three months It is possible that this large dose may have effaced some virulence differences between the strains (*Runyon* 39)

The growth of Davis strains at 4[illegible] *must also be emphasized* As mentioned previously growth at that temperature has been thought to be in favour of *M avium* and against Battey organisms avian like strains Group III (Battey) and non photochromogens (39 31 21 6 25) Our experiences with the above mentioned Battey strains from U S A support this However in other works no definite difference could be found in such growth experiments (10 40 4[illegible])

Other results in the present work place the Davis strains nearer Battey than *M avium*, viz the stronger catalase reaction (6 38) the *earlier growth* (21 39) and the displacement towards higher resistance to SM and lower resistance to INH (47 31 30 32 33)

It will be necessary to examine a larger number of Davis strains before the taxonomic relationship to *M avium* and Battey can be determined On the basis of existing information it must be assumed that Davis strains would be classified as *M avium* of perhaps slightly reduced virulence Differentiation between these types is of importance from epidemiological aspects

In the first part of the study brief mention is made of two strains serotype Yandle and Unclass special isolated from a heifer and from soil from a poultry run These strains were more different from the

typical pattern than Davis showing 24 and 22 divergencies respectively The results of the various examinations placed them outside the frame work of the present material

The *Yandle* strain was non pathogenic for rabbits and hens the colonies were orange in colour there was no growth at 43 and it gave a positive reaction to amides 3 5 and 6 According to *Bönicke* (7 8) the latter result excludes the possibility of the strain being classified as avian like This combination of positive reactions to three amides has been found previously in own experiments with strains possibly classifiable as *M scrofulaceum*

Unclass special was also non pathogenic for hens but it provoked marked joint processes in rabbits there was growth at 22 in the course of a week it gave positive reactions to amides 5 and 6 showed strong catalase reaction and (of particular interest) it gave strong positive reaction in the course of four hours in the Tween degradation test It is possible that the strain is identical with the *M terrae sp n* described by *Wayne* (45)

The strains in the previously published study of *M xenopei* (17) were also compared with the typical pattern of *M avium* The number of divergencies with those strains was between 19 and 27 that is to say definitely different from the typical pattern This is in agreement with the conclusions drawn in that study

The results of our examinations make it justifiable to conclude that *M avium serotype II* is the predominating cause of infections among hens cattle and swine in Denmark The sensitivity of the other animals from which such strains were isolated is stated by *Feldman* (19) to be mink readily infected sheep moderately susceptible horses highly resistant It is of special interest that all the strains from pigs were virulent as has been reported previously by *Christiansen* (9) *Plum* (35) and *Feldman* (18) Infections with attenuated strains as described by *Baumann et al* (3) *Kovacs* (21) and *Vallmann et al* (26) still do not occur in Denmark Serotyping of Kovacs s strains has been performed by Schaefer (*Kovacs* (22)) who demonstrated that the infections were caused by different serotypes

M avium as the cause of infections in animals in Denmark has been reported previously in a number of works mainly from Statens Veterinære Serumlaboratorium (for references see *Feldman* (18) and *Plum* (36)) and studies from recent years have shown that such infections still present problems (1 37) According to *Schaefer* (42) Av II is the only serotype that causes epidemics among hens as is also evident from the Danish material Furthermore since that type dominates the findings among the animal isolates it is justifiable to regard the infected hens and the soil contaminated by them as the main sources of infection

There were also Av II strains among the human isolates but in the present material it was found that there were equally many cases both

pulmonary and extrapulmonary caused by six other serotypes (mainly Davis).

It should be mentioned that the three patients in the previously published study (13) have been shown to be *infected by* New type (case 1) and Davis (cases 2 and 3). It is therefore quite probable that they were not infected directly by the hens. The assumption that case 1 was not the source of infection for the two other cases is confirmed by the serological findings.

Since serotypes must be considered to be constant characteristics (*Schaefer* (41)), *infections in man with the various serotypes other than* Av II have no epidemiological relationship to infections in animals. *Thus it may be expected that these infections will continue to occur in* Denmark despite eradication of avian disease among the animals.

SUMMARY

This work is a bacterial study of *M. avium*: 196 strains *isolated in* Denmark from animals and 22 strains from Danish patients.

The animal isolates originated mainly from *pigs, cattle and hens*. Only two of these strains failed to show definite alliance with *M. avium*. *As regards the other strains there were only minor irregularities between* the animal groups in respect of the distribution of results within the features examined.

The typical pattern for *M. avium* isolated from animals is described on the basis of 74 features: 40 from *in vitro* and 34 from *in vivo* experiments. Serotyping of a representative part of this material showed that serotype Av II predominated. As this probably also applies to all the animal isolates, the typical pattern presumably also describes serotype Av II.

Among the 22 human isolates seven different serotypes were found with Av II and Davis predominating. Based on the whole material some distinctive characterization of serotypes Av II and Davis was possible.

From the distribution of divergencies from the typical pattern it can be predicted that a strain with more than six divergent features will be *found to be of a serotype other than* Av II (or Av I). All the Davis strains showed from 10–19 divergencies.

It can further be assumed that *infected* hens and the *soil* contaminated by them may be the main source of the infection of animals. Serotype Av II was also found among *the human isolates, but in addition* there were six other types. These latter infections would appear *to have no* epidemiological relationship to animal infections and may thus be expected to continue to occur in Denmark despite eradication of avian disease among animals.

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Since completion of the manuscript Dr Schaefer has notified that the Battey strains from U S A referred to on page 308 consist of the following serotypes Chan (two strains *Darden* one strain Boone one strain *unclassified two strains*

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PARAMETERS IN 7α DEHYDROXYLATION OF BILE ACIDS BY ANAEROBIC LACTOBACILLI

By

TORE MIDTVEDT[1] and ARNE NORMAN

Received 10 viii 67

The microbial 7α dehydroxylation of bile acids plays an important role in the metabolism of these compounds both in man and animals (1) This reaction has been shown to be carried out by several strains of anaerobic bacteria isolated from rat and human faecal samples (9) These strains probably belong to the tribe *Lactobacilleae* (11) and are the only bacteria so far isolated capable of 7α dehydroxylation *in vitro* The isolated strains capable of 7α-dehydroxylation of chenodeoxycholic acid were also able to oxidize the hydroxyl groups at C-3 and C-7 to keto groups

One of the 7α dehydroxylating strains Strain II was selected for further study of bile acid transformations This report describes studies of the following phenomena—7α dehydroxylation of chenodeoxycholic acid in different culture media effect of pH on the reaction influence of incubation time on oxidation of the hydroxyl groups at C-3 and C-7 and on removal of the 7α hydroxyl group presence in washed cells and cell extracts of enzymes capable of performing these reactions and the nature of the metabolites formed in cultures originally containing either conjugated bile acids or free bile acids

MATERIALS AND METHODS

Bacteriological Procedures

Strain The bacterial strain used Strain II had previously been isolated from rat faeces (9) and tentatively identified as an anaerobic lactobacillus (11)

Culture technique Unless otherwise stated the cultures were grown in Todd Hewitt

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Bile Acids and Steroids No 1?

The following systematic name are g[illegible] mp[illegible] fe[illegible]ed to in this report by trivial names cholic acid 3α 7α 1[illegible] [illegible] 53 h [illegible] d ch[illegible]nodeoxycholic acid 3α 7α dihydrox[illegible]β-cholan[illegible] [illegible] 3α [illegible] α dihydroxy 5β-cholanoic acid lithocholi[illegible] acid 3α m[illegible] [illegible] noic acid

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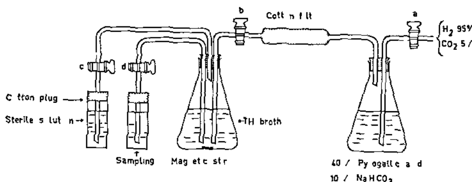


Fig 1
Apparatus for *sampling of batch cultures For explanation see text* (p 314)

broth (Oxoid) (TH broth) and incubated at 37 C for 7 days The inocula used were 0 01 ml of a 7 day TH broth culture in 5 ml of the same medium Anaerobic con ditions were obtained using the pyrogallol method after heating and cooling of the medium (9)

Sampling of batch culture In these experiments a simple batch culture system was used (Fig 1) The apparatus with the culture fl sk containing 495 ml of TH broth with 100 μM chenodeoxycholic acid was sterilized by autoclaving and the broth was inoculated with 0 5 ml of a 7 day TH broth culture of Strain II Stopcocks a b and c were opened and a mixture of 95 per cent H and 5 per cent CO was bubbled through the TH broth for 15 minutes to obtain anaerobic conditions in the culture flask Trace amounts of oxygen were removed by slowly passing the gas mixture through a solution of equal amounts of 40 per cent pyrogallic acid and 10 per cent $NaHCO_3$ in water After inoculation the apparatus was kept at 37 C At intervals aliquots of the broth culture were withdrawn while maintaining anaerobic con ditions in the culture flask by opening the stopcocks a b and c to let the gas mix *ture bubble through the broth* Stopcock c was closed and d opened After desired amounts of the TH broth had been collected in the sampling tubes stopcocks a b and d were closed A magnetic stirrer was used for 5–10 minutes before the samples *were withdrawn to get homogeneous samples During the experiment the samples* from the culture flask were checked for the presence of microorganisms other than Strain II

Chemical Procedures

24-C14 labelled bile acids Lithocholic chenodeoxycholic deoxycholic and cholic acids were prepared according to the method of *Bergstrom et al* (3) Conjugated bile acids were synthesized by the methods of *Bergstrom & Norman* (? 15) 3α hydroxy 7 keto 5β cholanoic acid was prepared according to *Samuelsson* (17) and 3 keto 5β cholanoic acid as described by *Norman & Palmer* (16)

Unlabelled bile acids Sources and references for synthesis of derivatives of cheno deoxycholic acid are given in an earlier publication (9) Derivatives of deoxycholic acid (6 7) were kindly supplied by *P Eneroth & J Sjovall*

Extraction methods After incubation the free bile a ids were extracted with ethyl *acetate from the broth* following acidification to pH 1 with hydrochloric acid The conjugated bile acids were extracted with butanol The ethyl acetate or butanol ex tracts were evaporated dissolved in acetone and aliquots were taken for chromat graphic analysis

Chromatographic technique The phase systems used for chromatography are listed in Table 1 Columns for reversed phase partition chromatography consisted of 4 ml of stationary phase supported on 4 5 g of hydrophobic Hyflo Super Cel (Johns Manville & Co USA) Two ml *fractions were collected and titrated with 0* NaOH in methanol

Aluminium oxide chromatography of the bile acid methyl esters was carr

TABLE 1

Chromatographic Systems

Phase system	Moving phase	Milli litres	Stationary phase	Milli litres
C1(14)	Methanol water	150 150	Chloroform isooctanol	15 15
F1(18)	Methanol water	165 135	Chloroform heptane	45 5
S10(5)	Trimethylpentane isopropyl alcohol acetic acid	60 20 0 5		
S12(5)	Trimethylpentane ethyl acetate acetic acid	5 25 0 2		
S15(5)	Trimethylpentane ethyl acetate acetic acid	20 20 0 2		
I(8)	n Butanol water acetic acid	50 5 5		

with aluminium oxide grade III (Woelm Eschwege Germany) eluting with increasing concentrations of benzene in hexane or ethyl acetate in benzene (4)

Thin layer plates were prepared using a suspension of 53 ml of distilled water and 30 g of kieselgel G (Merck, Darmstadt Germany) The chromatoplates were activated in an oven at 110 C for 1 hour before use Spots were revealed by spraying with concentrated sulphuric acid and charring at 250 C

Isotope determinations The isotope content in liquid samples was determined by plating aliquots and counting with a Frieseke Hoepfner methane gas flow counter After thin layer chromatography (TLC) radioactive spots were located by auto radiography For quantitation the optical density of the film was measured at 550 mμ

RESULTS

1 *Formation of Lithocholic Acid in Various Media*

Strain II was cultivated in 10 media to investigate the formation of lithocholic acid from chenodeoxycholic acid (Table 2) In 8 out of the 10 media the formation of lithocholic acid could be demonstrated Thus even though growth occurred in the remaining media this could not be correlated with lithocholic acid formation

2 *Effect of Variation in pH on Growth and Lithocholic Acid Formation in TH Broth*

As is evident from Table 3 no growth [illegible] with initial pH values of 5 0 and 5 5 At pH 6 [illegible] obtained but there was no formation of lithocholic acid In [illegible] with pH values between 6 5 and 8 0 moderate to abundant growth was obtained and lithocholic acid was formed Keto derivatives were formed at all the pH values studied Enzymes in the inoculum were responsible for the appearance of metabolites in media where no growth occurred

TABLE 2

Formation of Lithocholic Acid after Cultivation of Strain II in Various Media

Medium	Initial pH of medium	After 7 days incubation at 37 °C: pH of inoculated medium	pH of uninoculated control medium	Growth	Formation of lithocholic acid
Todd Hewitt broth (Oxoid)	8.05	7.25	7.30	++	+
Tryptone Soya broth (Oxoid)	7.40	6.40	6.95	+	+
Trypticase Soy broth (B.B.L.)	7.35	6.40	6.95	+	+
Bacto Brain Liver Heart medium (Difco)	7.30	6.30	7.10	++	+
Bacto Fluid Thioglycollate medium (Difco)	7.05	6.30	6.70	++	+
Bacto Lenassay broth (Difco)	6.95	6.65	6.75	+	+
Bacto Micro Inoculum broth (Difco)	6.65	5.50	6.45	++	+
Bacto Folic Acid Assay medium (Difco)	6.50	6.30	6.40	(+)	—
Bacto Folic Acid Assay medium (Difco) + 0.5 μg folic acid/ml medium	6.50	5.30	6.40	++	—
Bacto Folic Acid Assay medium (Difco) + 0.5 μg folic acid/ml medium + phosphate buffer (1/15 M, pH 7.2)	7.20	6.00	7.00	++	+

The media originally contained 10 μM labelled chenodeoxycholic acid and were inoculated with 0.01 ml of a 7 day TH broth culture of Strain II. After seven days of incubation the labelled compounds were analysed with TLC for formed lithocholic acid.
++ moderate to abundant growth; + slight growth; (+) hardly visible growth

TABLE 3

Effect of Variation in pH on Growth and Lithocholic Acid Formation of Strain H

Initial pH of medium	After 7 days incubation at 37 C				
	pH of medium	Growth	Unchanged chenodeoxycholic acid	Metabolites with hydroxyl or keto group at C-7	Metabolites without hydroxyl or keto group at C-7
			%	%	%
5.0	5.0	—	86	14	0
5.5	5.5	—	82	16	0
6.0	6.0	+	78	22	0
6.5	6.5	++	11	9	80
7.0	7.0	++	21	17	62
7.5	7.2	++			
8.0	7.3	++	19	25	56

TH broth was adjusted with 1 N NaOH or 1 N HCl to the pH selected and phosphate buffer (final concentration of 1/15 M) of the same pH added Sterilization was by filtration through a Millipore Filter Type GS 5 ml of broth was transferred to sterile tubes containing labelled chenodeoxycholic acid to give an original concentration of 10 μM The inoculum was 0.1 ml of 7 day cultures of Strain H in TH broth and incubation was at 37 C for seven days

— no visible growth + slight growth ++ moderate to abundant growth

3 *Effect of Incubation time on Transformation of Chenodeoxycholic Acid*

In batch cultures (Fig 1) keto derivatives were formed rapidly from chenodeoxycholic acid (Fig 2) Half of the original amount of chenodeoxycholic acid was transformed into keto derivatives within 24 hours Lithocholic acid could be demonstrated when visible growth occurred Therefore in all following experiments analyses transformation were made after 7 days of incubation

4 *Presence of Enzymes Capable of Transforming Chenodeoxycholic Acid in Penicillin Treated Cultures Resting Cells and Cell Extracts*

The results of experiments shown in Fig 3 show that a penicillin treated culture contains large amounts of enzymes capable of transforming chenodeoxycholic acid TLC analyses of the metabolites formed indicated that they consisted mainly of 3α hydroxy 7 keto 5β cholanoic 7α hydroxy 3 keto 5β cholanoic and 3 7 diketo-5β cholanoic acid No enzymes capable of removing the hydroxyl group at C-7 could be demonstrated

The location of the enzymes capable of oxidizing the hydroxyl groups at C-3 and C-7 to keto groups was studied in the experiments reported in Table 4 The enzymes were mainly present intracellularly and only slight activity was found in the sterile filtrate of the culture The

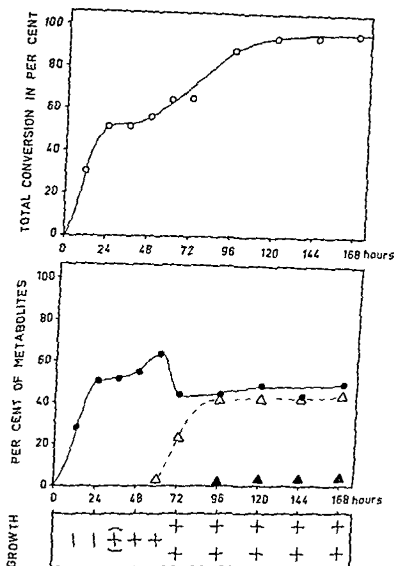


Fig 9

Change in composition of metabolites during incubation of Strain II in a medium originally containing 100 μM chenodeoxycholic acid -24 C14

Upper part Total conversion of chenodeoxycholic acid

Middle part Formation of metabolites of chenodeoxycholic acid ●—● metabolites with hydroxyl or keto group at C-7 △- - -△ lithocholic acid ▲ ▲ 3 ket 5β-cholanoic acid

Lower part Bacterial growth — no visible growth (+) hardly visible growth + slight growth + + moderate to abundant growth

washed resting cells were highly active and after sonification the enzyme activity was present in a soluble state The hydroxyl group at C-7 was always oxidized to a greater extent than that at C-3 No appreciable formation of 3 7 diketo 5β cholanoic acid was detected It

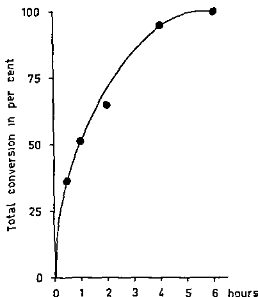


Fig 3

Aerobic transformation of chenodeoxycholic acid in penicillin treated cultures 5 ml of a 7 day TH broth culture of Strain II was transferred to a tube containing labelled chenodeoxycholic acid and penicillin G to give a concentration of 100 μM chenodeoxycholic acid and 1000 I U penicillin G/ml broth The tube was shaken aerobically at 37 C Samples (0 2 ml) were withdrawn 0 5 1 2 4 and 6 hours after the reaction was started and the samples were analysed for unchanged chenodeoxycholic acid

was observed that 0 01 ml of a broth culture (approximately the amount in the inoculation loop) contained a sufficient amount of enzymes to cause detectable oxidation of chenodeoxycholic acid in a 5 ml solution of phosphate buffer with a bile salt concentration of 10 μM

5 *Interconversion of Metabolites Formed from Chenodeoxycholic Acid*

The main metabolites formed from chenodeoxycholic acid by Strain II are 3α hydroxy 5β cholanoic 3 keto 5β cholanoic and 3α hydroxy 7 keto 5β cholanoic acids (9) These metabolites were incubated with Strain II The labelled metabolites in the broth were separated by TLC (Table 5) 3α hydroxy 5β cholanoic and 3 keto 5β cholanoic acid were not transformed into compounds with a hydroxyl or keto group at C–7 Dehydrogenation of the hydroxyl groups at C 3 and C–7 was found to be reversible However reductions of the keto groups at C–3 and C–7 only produced the 3α and 7α epimers 3α hydroxy 7 keto 5β cholanoic acid was transformed into compounds without a hydroxyl group at C–7 or reduced to 3α 7α dihydroxy 5β-cholanoic acid

TABLE

Presence in Penicillin Treated Cultures and Cell Extracts of Strain II

Sample	Material for studying enzyme activity Amount	Original chenodeoxycholic acid concentration	Results Unchanged chenodeoxycholic acid
		μM	%
Batch culture	0 5 ml	10	46
		100	79
Sterile filtrate of batch culture	0 5 ml	10	97
		100	100
Washed cells from batch culture	equivalent to 0 5 ml batch culture	10	57
		100	85
Cell free extract after sonification of the washed cells	equivalent to 0 5 ml batch culture	10	79
		100	91

A 7 day batch culture at 37 C of Strain II in TH broth was prepared from an original filtration through a Millipore filter type GS Washed cells were obtained by three subsequent cells was in MSE Ultrasonic Disintegrator (20 1 c 60W) operating at 1 5 A in 15 × 4 sec 20 minutes at 4 C followed by filtration of the supernatant through a Millipore filter from chenodeoxycholic acid after 2 hours of aerobic incubation Penicillin G was added to give a final concentratio

TABLE

Interconversion of Metabolites Formed fro

C14 labelled bile acid	Original bile acid concentration	chenodeoxycholic acid	Per cent 3β 7α dihydroxy 5β cholanoic acid	3α 7β dihydroxy 5β cholanoic acid
	μM			
chenodeoxycholic acid	10	f	—	—
	100	47	—	
3α hydroxy 7 keto 5β cholanoic acid	10	14	—	—
	100	61	—	—
lithocholic acid	10	—	—	—
3 keto 5β-cholanoic acid	10	—	—	—

6 *Metabolism of Deoxycholic and Cholic Acids*

A *Deoxycholic acid* Preliminary fractionation of metabolites of deoxycholic acid was obtained with reversed phase partition chromatography and the metabolites were separated into three groups (Fig 4)—Group I 3 12 dihydroxy 5β cholanoic acids—Group II 3 hydroxy 12 keto 5β cholanoic acids and 3 keto 12 hydroxy 5β cholanoic acids—

4
of En ymes Capable of Transforming Chenodeoxycholic Acid

after 2 hours incubation at 37 C				
	Per cent of metabolites with the TLC mobilites of			Metabolites without hydroxyl or keto group at C–7
3β 7α dihydroxy 5β cholanoic acid	3α hydroxy 7 keto 5β cholanoic acid	7α hydroxy 3 keto 5β cholanoic acid	3 7-diketo 5β cholanoic acid	
0	46	5	3	0
0	?0	1	0	0
0	2	1	0	0
0	0	0	0	0
0	37	5	1	0
0	13	2	0	0
0	15	5	1	0
0	6	3	0	0

culture of the same age in this medium A sterile filtrate of the culture was obtained by centrifugations and washings in phosphate buffer (1/15 M pH 7 4) Sonification of the *The soluble fraction was separated from the cell residue by centrifuging at 15 000 × g for* type CS The enzyme activity was investigated by determination of the metabolites formed at 37 C in 5 ml of phosphate buffer (1/15 M pH 7 4)
of 1000 I U /ml of incubation mixture

5
Chenodeoxycholic Acid by Strain II in " Day Cultures

metabolites with TLC mobilities of					
3α hydroxy 7 keto 5β cholanoic acid	7α hydroxy 3 keto 5β cholanoic acid	3 7 diketo 5β cholanoic acid	lithochol c ac d	3β hyd oxy 5β cholanoic a id	3 keto 5β cholanoic a id
4	—	—	80	—	10
??	1?	—	15	—	4
—	4	—	80	—	2
8	8	—	21	—	2
—	—	—	91	—	9
—	—	—	.7	—	73

Group III (retained in the stationary phase) 3 12 diketo 5β-cholanoic acid and d hydroxylated derivatives As shown in Fig 4 groups I and II contained labelled compounds No labelled compounds were retained in the stationary phase (Group III) Labelled compounds in Group II were separated by TLC into two fractions II a and II b (Fig 5) Aluminium oxide chromatography of labelled compounds in Groups I II a

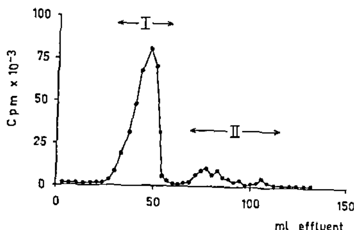


Fig 4

Deoxycholic acid metabolites produced by Strain II
Group column chromatographic separation of labelled metabolites in broth culture originally containing 10 μM deoxycholic acid –24–C^{14} Phase system F 1

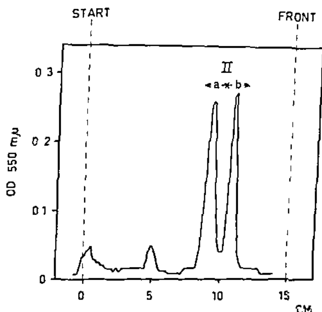


Fig 5

Separation of metabolites from fraction II (Fig 4) by TLC.
Phase system S12 Radioactive spots were detected by autoradiography and a densiometric recording of the autoradiograph is shown

and II b did not give further separation TLC of these fractions with phase systems S 10 S 12 and S 15 (Fig 6) revealed compounds with the TLC mobilities of—I unchanged deoxycholic acid—II a 3α hydroxy 12 keto 5β cholanoic acid—II b 12α hydroxy 3 keto 3β cholanoic acid Thus Strain II contains enzymes oxidizing the hydroxyl groups at C-3 and C-12

FRONT

START

3,12 - diketo

3α - OH, 12 - keto

3 - keto, 12α - OH

II b

II a

I

3β - OH, 12α - OH

3α - OH, 12β - OH

3α - OH, 12α - OH

Fig 6

Deoxycholic a id metabolites formed by Strain II TLC f gr up I (Fig 4) IIa III (Fig 5) and standard

Phase system S12 The black p ots represent rad oa t metab lite detected by aut ora liography and the cross hatched spots rej resent st ndard dete ted l pra ing with sulphuric acid Reference st ndards (3α OH 1? OH I h l id (3α OH 12β OH) 3α 12β dihydroxy 5β-ch olanoic a (3β (H)H I dihydroxy 5β cholanoic acid (3 keto 12α OH l k 3 x 3 k t an c acid (3α OH 1? ket) 3α hy lroxy 12 keto 5β ch t t 3 t 3 12 diketo 5β cholanoi)

B *Cholic acid* Met ibolites of cholic acid v s r l reversed phase partition chromatographs with phase s em (Aft r elution of the column with 200 ml of moving phas (I raction (I) the tri hydroxycholanoic acids is well as their keto de v tiv s were obt ined

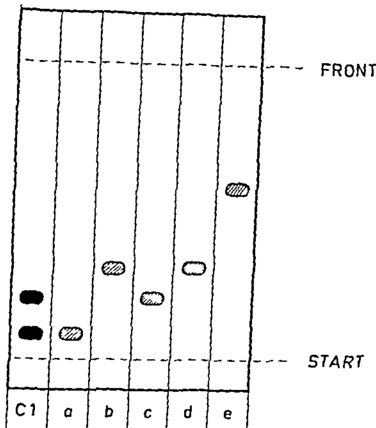


Fig 7

Cholic acid metabolites formed by Strain II TLC of group C 1 and standards Phase system S12 For explanation see Fig 6 Reference standards a cholic acid b deoxycholic acid c 3α 12α dihydroxy 7 keto 5β-cholanoic acid d 3α hydroxy 7 12 diketo 5β cholanoic acid e 3 7 12 triketo 5β cholanoic acid

Dihydroxycholanoic acids and their keto derivatives were retained in the stationary phase (Fraction C 2)

Labelled compounds in Fraction C 1 were separated with TLC (Fig 7) and appeared in the same zone as dihydroxymonoketocholanoic acids in addition to unchanged cholic acid No labelled compounds occurred in the position of monohydroxydiketo or triketocholanoic acids

Labelled compounds in Fraction C 2 were separated as described in section 6 A They were present in groups I II a and II b with different phase systems revealed the following compounds with TLC mobilities of—I 3α 12α dihydroxy 5β cholanoic acid—II a 3α hydroxy 12 keto 5β cholanoic acid—II b 12α hydroxy 3 keto 5β cholanoic acid Thus Strain II was able to 7α dehydroxylate cholic acid and to oxidize the hydroxyl groups at C-3 and C-12 of the deoxycholic acid formed

The effect of substrate concentration on the formation of cholic acid metabolites was studied and recorded in Table 6 In broth cultures

originally containing 10 μM cholic acid most of the isolated metabolites lacked hydroxyl or keto groups at C-7. In cultures originally containing 100 μM or more of cholic acid only a minor fraction of the isolated metabolites lacked hydroxyl or keto groups at C-7. The capacity of Strain II to oxidize the hydroxyl groups at C-3 and C-12 was very slight. The large amount of keto derivatives of cholic acid formed was due to the great capacity of Strain II to oxidize the hydroxyl group at C-7 as most of these keto derivatives had the TLC mobility of 3,12-dihydroxy-7-keto-5β-cholanoic acid.

TABLE 6

Concentration of Cholic Acid Metabolites in 7 Day Cultures of Strain II

Original concentration of cholic acid in TH broth	After 7 days incubation at 37°C			
	Concentration of unchanged cholic acid	Concentration of metabolites with TLC mobilities of		
		keto derivatives with hydroxyl or keto group at C-7	deoxycholic acid	12α-hydroxy-3-keto-5β-cholanoic acid, 3α-hydroxy-12-keto-5β-cholanoic acid
μM	μM	μM	μM	μM
10	0.5	0.1	9.2	0.2
100	25	44	27	4
200	50	99	47	4
400	172	162	64	2
600	297	240	63	0

Metabolites of labelled cholic acid were separated by column chromatography followed by TLC as described in section 6 B. The spots were located by autoradiography and the percentage distribution of labelled products was calculated from densiometric determinations of the film.

C. *Quantitative differences in the 7α-dehydroxylation of cholic and chenodeoxycholic acids.* Fig. 8 shows that cholic acid was dehydroxylated to a significantly higher extent than chenodeoxycholic acid. However, when both chenodeoxycholic and cholic acid were present the extent of dehydroxylation was the same for the two compounds.

7. *Metabolism of Tauro- and Glycocholic Acids*

No splitting of the conjugates was observed after incubation of taurocholic acid and glycocholic acid with Strain II but part of the bile acid conjugates had been transformed into less polar compounds. The incubation mixtures were hydrolyzed with 2 N NaOH for 24 hours at 135°C in a steel bomb and the free bile acids were analyzed with TLC. One half of the labelled compounds appeared at the position of unchanged cholic acid and the other half in one fraction with a TLC mobility of 3,12-dihydroxy-7-keto-5β-cholanoic acid.

Chenodeoxycholate-24-C^{14}	−	+	−	+	−	+
Cholate-24-C^{14}	+	−	+	−	+	−
Chenodeoxycholate μM	0	100	0	200	100	100
Cholate μM	100	0	200	0	100	100

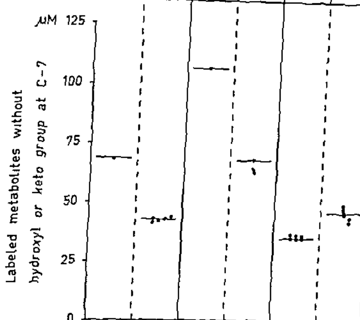


Fig 8
Quantitative differences in the 7α dehydroxylation of cholic and *chenodeoxycholic acids*
Series of 10 tubes containing either cholic acid (100 or 200 μM) or chenodeoxycholic acid (100 or 200 μM) or both acids (100μM each) were inoculated with 0 1 ml of the same culture of Strain II and were incubated for seven days

DISCUSSION

The results show that 7α dehydroxylation does not always occur in media supporting growth of Strain II (Table 2) and that it occurs only in media with pH above 6 0 (Table 3) TH broth extensively used in other investigations for detection of lithocholic acid (9 10 12 13) is characterized by a rather high initial pH (approximately 8 0) After Strain II has been cultured in this medium for 7 days the pH values are approximately 7 0 It would appear necessary however to buffer the medium to ensure the maintenance of a suitable pH for lithocholic acid formation by Strain II

Some of the pathways of chenodeoxycholic acid metabolism by Strain II are summarized in Fig 9 7α dehydroxylation was not reversible since no metabolites with keto or hydroxyl groups at C 7 were demonstrated in cultures originally containing 3α hydroxy 5β cholanoic or 3

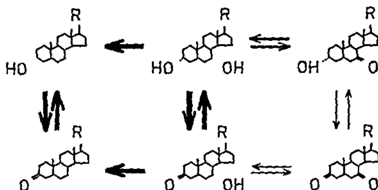


Fig. 9
Metabolism of chenodeoxycholic acid by Strain II

keto 5β cholanoic acid. Dehydrogenation of the hydroxyl groups at C-3 and C-7 was reversible. Reduction of the keto groups yielded only α hydroxyl groups.

The fact that Strain II is able to 7α dehydroxylate cholic acid has been established. When acting on cholic acid the dehydrogenases of Strain II were much more active in oxidizing the hydroxyl group at C-7 than that at C-3 or C-12. Thus cholic acid was mainly transformed into 3α, 12α dihydroxy 7 keto 5β cholanoic and 3α, 12α dihydroxy 5β cholanoic acid. The last mentioned metabolite was further transformed by oxidation of the hydroxyl groups at C-3 and C-12.

Analysis of the faecal bile acids in man has shown that the main microbial transformations are splitting of conjugates, elimination of the hydroxyl groups at C-7 and oxidation of hydroxyl groups at C-3, C-7 and C-12 to keto groups (1). Keto groups can be reduced to both α and β hydroxyl groups (1). Strain II could perform all these reactions except the splitting of conjugates and reduction of keto groups to β hydroxyl groups.

Since human bile normally contains large amounts of deoxycholic acid and only trace amounts of lithocholic acid, it was of interest to compare the capacity of Strain II to remove the 7α hydroxyl group from cholic and chenodeoxycholic acids. 7α-dehydroxylation was found to be of the same order of magnitude in cultures containing equal amounts of these two compounds. Therefore it is reason[illegible] similar degree of dehydroxylation of cholic and chen[illegible] this type of microorganism in the human intestine.

SUMMARY

A strictly anaerobic intestinal bacterium, Strain II, tentatively identified as a member of the tribe *Lactobacilleae*, was further studied for transformation of bile acids.

7α dehydroxylation was not found in all media supporting growth and was only present in media with pH above 6 0

Enzymes capable of oxidizing the hydroxyl groups at C–3 and C–7 were present in penicillin treated cultures but no enzymes capable of 7α dehydroxylation were demonstrated The oxidizing enzymes could be extracted from sonificated cells with phosphate buffer

Transformation of 3α hydroxy 5β cholanoic acid 3 keto 5β cholanoic and 3α hydroxy 7 keto 5β cholanoic acid showed that the keto groups at C–3 and C–7 were reduced but only to hydroxyl groups in α position

Transformation of cholic and deoxycholic acids showed that the 7α hydroxyl group of cholic acid could be removed and that oxidation of the hydroxyl groups at C–3 C–7 and C 12 occurred

No quantitative differences in removal of the 7α hydroxyl group from chenodeoxycholic acid and cholic acid occurred in cultures with equal concentrations of these acids

Taurocholic and glycocholic acids were not transformed into taurodeoxycholic or glycodeoxycholic acids

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THE SPECIFICITY OF CIRCULATING ANTIBODIES IN EXPERIMENTAL INFECTION WITH *MYCOBACTERIUM AVIUM* DEMONSTRATED BY IMMUNOFLUORESCENCE

By

J Bennedsen

Received 10 xii 67

The presence of antibodies in the serum globulin of experimental animals infected with virulent mycobacteria has not often been reported It has been considered that it is more difficult to prove the existence of such antibodies than of those caused by injection of killed mycobacteria or more or less purified mycobacterial antigens (*Boyden* 1958)

Takahashi et al (1961) compared several haemagglutination techniques and were able to demonstrate antibody activity in rabbits infected with *M tuberculosis* or *BCG* However as regards the question of specificity of the antibodies haemagglutination reactions are of no value when type specific antigens are not available

Using immunodiffusion Lind (1959 1960) demonstrated precipitating antibodies in rabbits infected with M tuberculosis The reactions were weak and were found late in the infection period In our laboratory we have not been able to reveal the presence of precipitating antibodies in serum from rabbits infected with *M avium or M bovis*

In a previous paper (*Bennedsen* 1966) it was reported that antibodies could be demonstrated by means of immunofluorescence in serum from rabbits infected with virulent mycobacteria It was shown that the antibodies produced early in the infection possessed a high degree of specificity *for the infecting agent In order to examine that* aspect further studies were performed with a collection of strains The present paper reports the demonstration by *means of immunofluorescence of nine different serotypes of M avium and avian like strains*

MATERIAL

Engbæk et al (1968a b) have examined a series of strains isolated mainly from cattle pigs hens and man Some of these strains were subjected *to serological typing* by *Schaefer (1965)* who *found nine* different serotypes in the collection by means of agglutination tests Nine strains were selected on the basis of his results and antisera against them were produced All the strains were then *tested against the nine* type sera

pretation of the findings mentioned above test strain Davis (SSC 1007) was found to react with both type serum IV and D

Table 3 shows the results of all 62 strains tested by both the indirect and direct techniques The strains are grouped and designated according to the results obtained by Schaefer (left hand column)[1] Only the test strain was present in the Av I group Of the 39 strains in the Av II group 38 gave a positive reaction with test serum II One strain did not react with any of the nine test sera but when the strain was used for

TABLE 1

Fluorescence Antibody Reactions with Nine Test Strains and Corresponding Nine Test Sera Using the Indirect Technique

(The figures in italics are the homologous reactions)

Test strain		Test serum								
Schaefer's serotype	Strain no	I	II	III	IV	D	W	Y	O	H
Av I	SSC 691	*4*	0	1	0	2	2	2	1	1
Av II	SSC 796	2	*4*	1	1	2	2	2	1	0
Av III	SSC 789	1	1	*4*	1	1	1	0	1	1
Av IV	SSC 315	2	2	1	*4*	0	2	2	1	1
Davis	*SSC 1007*	*0*	*1*	*1*	*0*	*4*	*0*	*2*	*1*	*1*
Watson	SSC 999	1	1	0	0	0	*4*	2	1	1
Yandle	SSC 637	0	1	1	0	0	0	*4*	1	1
New type	SSC 317	2	1	1	2	1	1	0	*3*	1
Howell	SSC 321	2	0	1	2	2	1	1	2	*4*

SSC = Statens Seruminstitut Collection

TABLE 2

Fluorescence Antibody Reactions with Nine Test Strains and Eight of the Corresponding Test Sera Using the Direct Technique

(The figures in italics are the homologous reactions)

Test strain		Test serum								
Schaefer's serotype	Strain no	I†	II	III	IV†	D	W	Y	O†	H
Av I	SSC 691	*3*	2	2	1	1	1	2	2	n e
Av II	SSC 796	1	*4*	1	2	2	1	2	2	n e
Av III	SSC 789	2	0	*4*	1	0	1	1	2	n e
Av IV	SSC 315	2	1	1	*3*	1	2	2	2	n e
Davis	SSC 1007	2	2	1	3	*4*	1	1	1	n e
Watson	SSC 999	2	0	1	1	0	*4*	1	1	n e
Yandle	SSC 637	1	1	1	1	2	1	*3*	1	n e
New type	SSC 317	1	2	1	2	1	2	2	*3*	n e
Howell	SSC 321	1	2	2	1	2	1	2	2	n e

SSC = Statens Seruminstitut Collection

† Conjugates were produced according to Procedure B (see text)

n e = not examined

[1] Our readings were performed without *prior knowledge* of these serotype

rect techniques in the Davis group Two strains reacted with both test serum IV and D, one of these being the test strain Davis

DISCUSSION

Schaefer (1965) found several serological groups of *M avium* and related strains by means of agglutination reactions Since we are not in a position at present to serotype by that method the results of Schaefer s determination of our collection of strains forms the basis of this study Thus it is not exactly the same cultures that were subjected to Schaefer s and our examinations Moreover the immunization procedures for production of antisera were different in the two systems Schaefer used hyperimmunization with heat killed phenolized mycobacteria while our sera were obtained from an early stage of the infection Some of the inconsistencies might thus be explained by this lack of parallelism However comparison of the results of immunofluorescence with those of Schaefer s agglutination test shows that it is possible to distinguish between serological types of *M avium* and allied strains by means of the fluorescence method It should be emphasized that none of the sera used for immunofluorescence typing was absorbed

I wish to stress that the results in Table 3 involve only the reaction between our test sera and the strains examined Interpretation of these results as regards the serotype of any particular strain is dependent on Schaefer s serotyping of the corresponding test strain The appearance of a strain or group of strains in Table 3 does not necessarily indicate that they can be designated as *M avium* or avian like strains

Our results are comparable with those of *Jones et al* (1965) who concluded that one of the major advantages of the immunofluorescence technique is the possibility of typing autoagglutinable and rough forms of mycobacteria We were in fact able to type some of such strains in Schaefer s unclassified group Another point of interest is the minute amount of antigen necessary for the immunofluorescence reaction It is possible to select single colonies from a plate and investigate rapidly the serological significance of any variations One of the strains examined gave inconsistent reactions when taken from Lowenstein Jensen tubes The strain was then cultivated on oleic acid agar plates and gave growth of a rough and a smooth form The smooth form gave positive reaction to test serum II and the rough form gave no reaction to any of the nine test sera

Compared with other serological methods both the immunofluorescence and the agglutination procedures seem to be specific Both methods utilize the surface antigens for their reactions and it seems probable that the type specific antigens are located on the surface of the cell Further serological investigation on the morphological basis is required to prove this assumption

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	ILEUM	CECUM	FECES	
M t b l m	— — — —	+ + + + + — —	+ + + — —	El m t f 7α OH
	— — —	+ + + + + +	+ + + +	3α OH → 3 k t
f	— — — —	+ + + + + +	+ + + +	7α OH → 7 k t
bl d	+ + +	+ + + + + + + +	+ + + + + + +	Splt tt g f t j g t
	+ +	+ + + + + + +	+ + + +	Spl tt g f gly m j g t
t l l g wth				A b
				b
	3 4 5 6 7 8	3 4 5 6 7 8 9 10 11 12	3 4 5 6 7 8 9 10 11 12	

D l t t p 1/l g 10

Fig 1

ıt No 1 Microbic bile acid transformation and bacterial growth in dilutions of rat intestinal content from ileum caecum and from faeces

	ILEUM	CECUM	FECES	
M t b l m	— — —	+ — — — — —	+ — — — —	Elim t f 7α OH
	— —	+ + + + + + +	+ + + + + + + —	3α OH → 3 k t
f	— — —	+ + + + + + + —	+ + + + + —	7α OH → 7 k t
bl d	— — —	+ + + + + + +	+ + + + + + + +	Spl tt g f t r j g t
	— — —	+ + + +	+ + + + + +	Spl tt g f gly j g t
t t l g wl				A b
				A b
	3 4 5 b 7 8	3 4 5 6 7 8 9 10 11 12	3 4 5 6 7 8 9 10 11 12	

D l t t p l 1/l g 10

Fig 2

at No 2 Microb c bile acid transformation and bacterial growth in dilutions of rat intestinal content from ileum caecum and from faeces

	ILEUM	CECUM	FECES	
M t bol m	— —	— — —	+ — —	El l f 7α OH
	—	+ + + —	+ + —	3α OH → 3 k t
	+ —	+ + + — —	+ + —	7α OH → 7 t
bl d	+	+ +	+ + +	Spl fl g f t ne j g t
	+ + +	+ + + + +	+ + + +	Spl tt g f gly ne j g t
B t l g th				A b
				b
	3 4 5 6 7 8	3 5 6 7 8 9 10 1 2	3 4 5 6 7 8 9 10 11 12	

D l t t p 1 l g 10

Fig 3

Rat No 3 M crobic bile acid transformation and bacterial growth in dilutions of rat intest nal content from ileum caecum and from faeces

In the ileum the total number of microorganisms was 10^9–10 and these values were obtained under both aerobic and anaerobic conditions In the caecum content the total number of bacteria was 10 –10^{11} These values were obtained under anaerobic conditions whe eas under aerobic conditions they were lower 10^7–10^9 Under anaerobi conditions the total number of microorganisms present in the faeces was 10^9–10^{10} and under aerobic conditions 10^7–10^9

TABLE

A Comparison of the Properties of the 10 Isolated

Test performed	Strains No 1	2	3
Microscopy	Gram neg rods	Gram neg rods	Gram neg rods
Uniform turbidity in TH broth	+	+	+
Motility	—	—	—
Gas in TDI medium	+	+	+
Fermentation of §			
L arabinose	—	—	—
Dextrose	Aw	Aw	Aw
Lactose	Aw	—	—
Litmus milk	—	—	—
Liquefaction of gelatin	—	—	—
Production of H_2S	+	+	—
Production of *indol*	+	+	+
Survival after exposure to			
70 C 20 minutes	—	—	
aerobic culture conditions 24 hours	—	—	

The sign (+) = *positive results obtained in the test*
TDI medium Difco *Trioglycollate Medium without*
§ (Aw) = *pH between 6 00–6 50 (A) = pH below 6 00*

Transformation of Bile Acids in Cultures from the Anaerobic Dilution Steps

Splitting of taurocholic and glycocholic acids and transformation of labelled chenodeoxycholic acid were determined in anaerobic TH broth cultures from all the dilution steps The metabolites formed were separated by thin layer *chromatography* (TLC) with different phase systems The results are summarized in Figs 1–3 (upper parts) Removal of the 7α hydroxyl group was demonstrated by isolating the metabolites lithocholic acid 3β hydroxy 5β cholanoic acid and 3 keto 5β *cholanoic acid Oxidation of the hydroxyl group at C–3 to a keto group gave rise* to the metabolites 7α hydroxy 3 keto 5β cholanoic acid 3 7 diketo 5β cholanoic acid and 3 keto 5β cholanoic acid and oxidation of the hydroxyl group at C–7 was evidenced by the presence of the metabolites 3α hydroxy 7 keto 5β cholanoic acid 3β hydroxy 7 keto 5β cholanoic acid and 3 7 diketo 5β cholanoic acid

Removal of the 7α hydroxyl group Elimination of the 7α hydroxyl group did not occur in the cultures of microorganisms from the ileum Microorganisms capable of removing the 7α hydroxyl group were always demonstrated in the caecum content and faeces The number of organisms in the caecum content and the faeces in one and the same rat seemed to be of the same order of magnitude whereas wide variations in the number of organisms present in the different rats were observed (10^{3}–10^{5}) The microorganisms capable of the 7α dehydroxy

2
Strains Capable of Splitting Bile Acid Conjugates

			Strains No			
4	5	6	7	8	9	10
Gram neg rods	Gram neg rods	Gram pos rods	Gram pos rods	Gram pos rods	Gram pos rods	Gram pos rods
+	+	+	+	+	+	+
—	—	—	—	—	—	—
+	+	—	—	—	—	—
—	—	A	A	A	A	A
Aw	Aw	A	A	A	A	A
—	—	A	A	A	A	A
—	—	Acid clot	Acid clot	Acid clot	Acid clot	Acid clot
—	—	—	—	—	—	—
—	+	—	—	—	—	—
+	+	—	—	—	—	—
—	—	—	—	—	—	—
—	—	+	+	+	+	+

performed and the sign (—) = negative results
Dextrose or Indicator
(—) = pH above 6 50 in the TDI medium

lation of bile acids do not seem to be among the quantitatively dominant anaerobes in the intestinal tract

In order to ascertain if the culture conditions were favourable to 7α dehydroxylation the transformation of chenodeoxycholic acid was tested in cultures from all the dilution steps added to broth cultures of a previously isolated anaerobic lactobacillus Strain II capable of performing this reaction (9) 7α dehydroxylation was observed in all the cultures

Oxidation of hydroxyl groups at C-3 and C-7 The hydroxyl group at C-3 was oxidized in cultures of the ileum content from all three rats (dilution steps 10^{1}–10^{4}) and the hydroxyl group at C-7 was oxidized in two rats (dilution steps 10^{3}–10^{4}) In cultures of the caecum content and faeces oxidation of these two hydroxyl groups could be demonstrated at higher dilution steps (10^{7}–10^{11}) in all the rats

Splitting of conjugates In two out of the three rats investigated deconjugating microorganisms were present in the hi he dilution steps of the ileum content that showed growth In the hird at the number of deconjugating microorganisms p esent was 10 hereas growth occurred up to dilution step 10^{6} In the caecum ont nt of all three rats deconjugating microorganisms re presen i the highest dilution steps showing growth Similar r sults wer obtained in the faeces from two of the rats and in the third no plitting of conjugates was observed in the highest dilution step showing growth

Isolation of microorganisms capable of splitting conjugated bile acids From the anaerobic cultures of the last dilution steps showing deconjugation single strains of *microorganisms* were isolated and tested for their ability to split bile acid conjugates Ten out of 17 strains of anaerobic microorganisms isolated from the ileum content of one rat and from the caecum content and faeces of all three rats were capable of performing this *reaction These* active strains labelled 1–10 were selected for further identification procedures The data presented in Table 2 indicate that the Gram negative strains 1–5 might be members of the family *Bacteroidaceae* and the Gram positive strains 6 10 members of the *tribe Lactobacilleae*

Strains 1–10 were tested for their ability to transform free bile acids by subculturing them in TH broth originally containing either 10 μM cholic acid 24 C^{14} 10 μM chenodeoxycholic acid 24 C^{14} or 10 μM lithocholic acid 24 C^{14} All the *Gram negative strains* were capable of oxidizing the hydroxyl group at C–7 to a keto group whereas none of the Gram positive strains was able to transform the free bile acids (Table 3)

TABLE 3

The Metabolism of Free Bile Acids by Isolated Microorganisms Capable of Splitting Conjugated Bile Acids

Strain No	The TLC mobilities of metabolites formed from		
	lithocholic acid	chenodeoxycholic acid	cholic acid
1	0	3α hydroxy 7 keto 5β cholanoic acid	dihydroxymonoketo derivative of *cholanoic acid*
2	0		
3	0		
4	0		
5	0		
6	0	0	0
7	0	0	0
8	0	0	0
9	0	0	0
10	0	0	0

DISCUSSION

Bacterial Counts in Intestinal Contents

One of the difficulties in estimating the quantity of microorganisms in intestinal contents is the use of suitable media The choice of TH broth used in this investigation was because this medium has been extensively used by us in studies of microbial bile acid transformation (7 8 9 10) and is able to sustain growth of microorganisms transforming bile acid in undefined mixtures of faecal microorganisms (7) and of strains belonging to 18 genera normally found in the intestinal contents of man and rat (10)

The results given in Figs 1–3 show a predominance of microorganisms grown under anaerobic conditions over those grown aerobically Such predominance has been previously emphasized by several investigators (14 15) These anaerobic microorganisms are found to belong to the groups of bacteroides and lactobacilli (14 15) The microorganisms isolated in this investigation may be included in these two groups

Microorganisms Transforming Bile Acid

The microorganisms that account for the 7α dehydroxylation were not shown to be present in the ileum content but a small number were found in the caecum content and the faeces Ability to cause 7α dehydroxylation had been previously observed in anaerobic lactobacilli isolated from human and rat faecal samples (7) but could not been demonstrated in 55 strains belonging to genera normally found in human and rat intestinal contents Hence this reaction which is a major feature in the intestinal bile acid transformation seems to be a rare property in intestinal microorganisms and is present only in organisms found in the caecum content and faeces

The demonstrated ability of the isolated strains in the group of bacteroides to oxidize the hydroxyl group at C–7 but not at C–3 is in agreement with previous findings in strains belonging to this group (10) The isolated strains of anaerobic lactobacilli capable of splitting bile acid conjugates failed to have any effect on the hydroxyl groups at C–3 and C–7 Ability to oxidize these hydroxyl groups varies in strains belonging to this group of microorganisms (10)

It was shown that the isolated strains of microorganisms capable of splitting bile acid conjugates can be included in the family *Bacteroidaceae* and the tribe *Lactobacilleae* Ability to split conjugated bile acids has previously been demonstrated in many strains belonging to these two groups of microorganisms (10)

Thus in contrast to the removal of the 7α hydroxyl group the ability to oxidize the hydroxyl groups at C–3 and C–7 and to split conjugated bile acids is found among the most common anaerobic intestinal microorganisms However it is reasonable to assume that the intestinal contents include several types of microorganisms capable of transforming bile acids and present in varying numbers Therefore it is of interest to ascertain which types of microorganisms are of importance for the main transformations of bile acid occurring *in vivo* This can be investigated by contamination of germfree rats with microorganisms the effect of which on bile acids *in vitro* is known

SUMMARY

Bile acid transformations were studied in anaerobic cultures from dilution steps of the intestinal content from the small intestine caecum and

faeces from three rats. No microorganisms capable of the 7α dehydroxylation of bile acids were observed in the contents of the small intestine; they were present, however, although in varying numbers (10^4–10^8), in the caecum content and the faeces. Anaerobic microorganisms capable of splitting conjugates and oxidizing the hydroxyl groups at C-3 and C-7 to keto groups were present in large numbers.

From the last dilution step where hydrolysis of conjugates occurred 10 strains able to split conjugated bile acids were isolated. Five of these strains, tentatively identified as members of the family *Bacteroidaceae*, could oxidize the hydroxyl group at C-7 to a keto group. The other 5 strains, which might be included in the tribe *Lactobacilleae*, were not able to transform free bile acids.

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freezing and thawing The supernatant was again the immunization antigen The rabbits were inoculated 5 times subcutaneously with 0 2 to 0 3 ml of the antigens at two week intervals First and second injections were given with Freund s complete adjuvant Serum specimens were obtained at times indicated in Table 1

The sera were tested for CF antibody against cytomegalovirus herpes simplex virus and uninoculated control fibroblast antigens using Takatsy Sever micromethod (Sever 196?) Antigens for CF tests were prepared by freezing and thawing virus infected fibroblasts at the time of complete cytopathic effect (viral antigens) or uninfected fibroblasts (control antigen) in half a volume of maintenance medium All CF antigens were used at 1/2 dilution Tested against positive human sera cytomegalovirus as well as herpes antigen contained at this dilution four antigen units For removing antibody to normal fibroblast components serum specimens diluted in 1/4 were absorbed at 4 C overnight with one fifth volume of washed packed fibroblasts For neutralizing antibodies the sera were tested with plaque reduction neutralization technique (Plummer & Benyesh Melnick 19 4)

Results of the CF tests are shown in Table 1 With both immunization antigens quite satisfactory homologous CF titres were obtained The rise of antibody was quite prompt a high titre being detected in three weeks prolongation of the immunization was not advantageous As could be expected CF antibody against control fibroblast antigen was also formed especially in Rabbit No 1 immunized with frozen and thawed antigen In Rabbit No 2 this undesirable antibody was at a slightly lower level The titres against herpes virus were similar to those against control fibroblast antigen The five week specimens were tested after absorption with fibroblasts The absorption had no effect on the anticytomegaloviru titres The absorbed serum specimen of Rabbit No 1 had titres of 1/8 and 1/16 against herpes and control antigens but the specimen of Rabbit No 2 showed no antibody against these antigens at the dilution of 1/8

The results of neutralization tests were less encouraging (Table 2) The nine week specimens had some neutralizing antibodies but in rather low titres

TABLE 2

Neutralizing Antibodies in Rabbits Immunized with Two Alkaline Treated Cytomegalovirus Antigens

	Weeks of Immunization				
	0	3	5	7	9
Rabbit No 1	–	–	9_s	< 8	18
Rabbit No 2	<8	<8	<8	<8	53

Not tested Reciprocal 80 per cent plaque reduction titre

See the legend of Table 1

The results of the present experiments indicate that it is possible to produce CF antibodies against cytomegalovirus in rabbits by immunizing with alkaline extracted antigen Obviously alkaline treatment either releases from cells some viral antigen which is more immunogenic than those obtained by conventional methods or causes degradation of viral proteins thus unveiling some normally hidden immunogenic determinants The low neutralizing antibody titres might partly have been caused by an unfavourable infectivity particle ratio in the test virus stock It is also possible that antibody produced against alkaline treated viral antigens have only a weak neutralizing capacity

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BRIEF REPORT

IS THERE A RELATIONSHIP BETWEEN GIANT CELL ARTERITIS WITH POLYMYALGIA RHEUMATICA AND RHEUMATOID ARTHRITIS?

By *Erik Waaler & Einar-Johan Milde*

The syndrome which to day usually is called polymyalgia rheumatica has attracted special attention throughout the last 15 years (2 3 5 7 9 14 and 15) Recently some authors have noticed that a granulomatous arteritis often is connected with the condition (1 6 8 10 12 and 13) and the name polymyalgia arteritica has been coined (8) However *Bagratuni* (4) claims that none of his patients developed arteritis

The aetiology and pathogenesis of the condition are unknown but some authors have suggested its close relationship to rheumatoid arthritis (2 3 14) One recently observed patient presented changes which may throw some light upon the pathogenetic mechanism and our findings will be reported briefly

Case Report

An 83 year old woman was after a period with general malaise admitted to hospital with severe headache Both temporal arteries were swollen ESR 133 mm Westergren haemoglobulin 69 per cent *a* globulin increased rheumatoid factor in serum negative (Latex Waaler Ripley) A biopsy from the temporal artery showed a granulomatous arteritis affecting all layers and with typical giant cells In some areas fibrinoid necrosis was found together with a slight palisading tendency of the cells similar to the picture seen in rheumatoid granuloma This inspired us to try to demonstrate a rheumatoid factor in the lesion and we asked for fresh material A frozen section from one temporal artery was examined using the mixed agglutination technique (16) Sheep red cells sensitized by rabbit antiserum were used as indicator system (11) The sensitized cells adhered to the tissue section while non sensitized cells did not The reaction was blocked by aggregated gamma globulin. These findings indicate that rheumatoid factor is present in the diseased artery

Discussion

In this patient with granulomatous arteritis and general symptoms rheumatoid factor was present in the lesion but not in the serum The condition is therefore similar to the so called sero negative cases of rheumatoid arthritis In these patients the sensitized sheep cell reaction is negative in the serum but most of them have positive reactions in the diseased joint capsules *Milde & Tonder* (16)

The demonstration of rheumatoid factor in tissue section from the diseased artery indicates a relationship between rheumatoid arthritis and giant cell arteritis with polymyalgia rheumatica The name given to the condition by *Bagratuni* non arthritic *rheumatoid disease* appears to be adequate *The answer to our* question in the title therefore should be in the affirmative

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BRIEF REPORTS

A DOUBLING OF MORBIDITY FROM TESTIS CARCINOMA IN COPENHAGEN 1943-1962

By Johannes Clemmesen

In an analysis of English mortality data from 1911-1955 *Case* 1956 gave age curves for cancer of testes showing one broad peak formed by age groups between 30 and 55 years and a secondary rise following the 60th year He found no trends characteristic of successive cohorts of the population and grouped testes as showing no definite change in rates

Grumet & Mac Mahon 1958 studied mortality data for New York City from 1949-1954 for the United States for 1933-1938 and registry data for Connecticut 1935-1940 They confirmed *Case*'s age curve for white men as well as the observation by *Schrek* 1944 that mortality rates for Negroes show only the smaller old age peak This may be well in keeping with the extreme rarity of testis cancers reported from Africa (*Oettlé & Higginson* 1966) although fear of castration among primitive men should not be entirely disregarded as a possible factor However neither the data used by *Grumet & Mac Mahon* nor later U S data on morbidity (*Dorn & Cutler* 1959 *Eisenberg* 1960) allow the separation of testis neoplasms from those of Male Genitals excl Prostate but for this category *Grumet & Mac Mahon* found upward secular trends during 1933-1954 resulting in about 50 per cent higher rates for all age groups between 15 and 44 almost certainly accounted for by an increase in mortality from testis tumours

The joint international *UICC report* on morbidity data from various cancer registries from 1966 allows a coarse comparison of age curves for testis tumours It appears that most age curves are of similar shape but that differences in level mostly affect the 15-44 year peak so that e g Finland and Israel with a low number of cases approach the age distribution of mortality among U S Negroes

Danish morbidity data on testis carcinoma cover more cases than most if not all other units They cover a racially uniform population and in contradistinction to mortality data they are immune to the difference in prognosis between seminoma and embryonal carcinoma During the period 1943-1957 this site was unusual in showing higher rates for Provincial Towns than for the Capital this suggesting that other differences should be evaluated with some caution (*Clemmesen* 196) However recent analysis of the entire period 1943-1962 shows regular trends in good conformity with results reported from other countries although the doubling of morbidity rates in the Capital shows a more certain rise and higher rates than other results on record

Fig 1 illustrates morbidity rates for the Scandinavian countries as given in the joint UICC report Fig 2 showing outspoken urban rural differences in morbidity suggests the advantage of attention to variations in urbanization in such international comparisons

Table 1 by means of morbidity rates age adjusted for the U S Standard population of 1950 illustrates levels and trends for three habitation areas Capital Suburb being excluded The rise in the Capital seems to have occurred later and to have been steeper than in the Provincial Towns The constant and high percentages of hospital admissions and of histological verification tend to confirm that the increase is not due to changes in diagnostic procedure

On the whole our results underline that also malignant neoplasms in organs apart from the inner and outer surfaces of the body may be exposed to exogenous carcinogenic influence Studies are being continued

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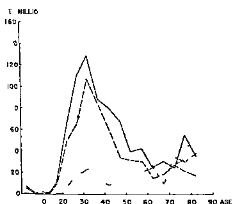


Fig 1

———	Denmark	1958–62	5?5 cases
— — —	Norway	19ɔ9–61	186 cases
------	Sweden	1959–61	?90 cases
	Finland	19ɔ9–61	71 cases
▲ ▲ ▲	Iceland	1955–63	15 cases

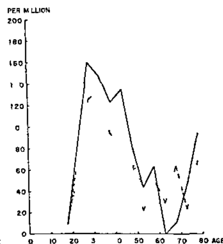


Fig 2

Testis Men Denmark 19ɔ8–62
——— Capital
------ Provincial towns
Rural areas

TABLE 1

Testis Carcinoma Danish Cancer Registry 19 3–1962 Cases and Incidence Rates per 100 000 Age Adjusted for the United States Standard Population of 19ɔ0

	Capital	Pro incial towns	Rural areas	Denmark
1943–47	80 (3 ?)	98 (3 8)	153 (3 0)	34?
1948–5?	86 (3 6)	10? (3 8)	169 (3 4)	374
1953–57	100 (4 3)	1 ɓ (4 6)	180 (3 7)	438
19ɔ8–62	134 (6 3)	147 (5 3)	174 (3 7)	5?ɔ

Per cent of Cases hospitali d and per cent *of these* verified by H tolog cal examination

	Capital		Provincial towns		Rural areas	
	In Hosp %	Hist Verif %	In Hosp %	Hist Verif %	In Hosp %	Hist. Verif %
1943–47	95	92	98	91	94	9?
1948–5	99	9ɾ	98	98	96	96
19ɔ3–57	99	9ɔ	97	86	97	94
1958–62	99	96	97	99	98	94

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BRIEF REPORT

VASCULAR PERMEABILITY CHANGES IN EXPERIMENTAL BRAIN CONCUSSION

A Preliminary Report

By *Lennart Rinder* and *Ingve Olsson*

The relations between the symptoms and lesions observed and the immediate mechanical course within the skull following blunt violence to the head are of great importance for the elucidation of the pathogenesis in head injuries Theoretical considerations have predicted and model and cadaver experiments have shown that pressure changes of a few milliseconds duration regularly occur within the skull if blows to the head occur (*Sjövall* 1943 *Sellier & Unterharnscheidt* 1963 *Lindgren* 1966 *Thomas et al* 1967) These pressure changes may produce deformations of the brain tissues (*Gurdjian & Lissner* 1961) —*Vascular factors are of considerable importance in head injuries* (c f *Langfitt et al* 1966) Increase of vascular permeability is a major factor in the development of brain oedema (*Klatzo* 1967) We have studied vascular permeability changes using fluorochrome labelled albumin as indicator in experimental brain concussion produced by brief and well defined pressure pulses within the skull similar to pressure changes recorded in experiments with blows to intact cadaver skulls (*Lindgren* 1966)

Material and Methods

Experiments were performed on 24 rabbits —In a parietally trephined hole (diam 17 5 mm) a tight fitting plexiglass tube was applied and connected to a closed plunger system The whole system was completely filled with physiol saline By hitting the plunger pole a predictable and measurable fluid volume (0 2 0 4 ml) was suddenly introduced extradurally into the skull cavity Thus a brief pressure pulse was generated within the skull contents *The pressure pulse was measured by* a transducer near the brain surface and recorded on an oscilloscope The duration of the pressure pulse was 5–15 milliseconds and the peak amplitude varied from 0 5 to 2 5 atm

Through a catheter introduced into the aorta the blood pressure and the heart rate were continuously recorded. The respiratory movements were recorded and the conditions of some reflexes were observed

Increase of vascular permeability with formation of brain oedema was studied by observing the distribution of bovine albumin labelled with Evans Blue (EBA) (*Steinwall & Klatzo* 1966) EBA was injected into the aorta 15 minutes before the *induction of the pressure pulse* or at varying times thereafter The animals were *sacrificed immediately* or some hours *after the pressure pulse and the* CNS fixed by perfusion through the catheter with 10 per cent formalin Frozen sections (10 my) from several levels of the brain and cervical cord were examined in a fluorescense microscope and EBA traced by its intense red fluorescense (*Steinwall & Klatzo* 1966 *Hamberger & Hamberger* 1966)

12 rabbits served as controls and were subjected to all above mentioned procedures except for the induction of the pressure pulse

Results

In the controls EBA was never observed outside the blood vessels except for the previously wellknown areas (*Klatzo et al* 1967) —Following the induction of an

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Fig 1

a Exudation of labelled albumin (white areas) within the lateral part of the medulla oblongata

b Nerve cells with uptake of labelled albumin within the lateral part of the medulla oblongata

intracranial pre sure pulse with a duration of 5–15 milliseconds and with peak amplitude exceeding 1 0 atm vascular permeability was increased and FBA penetrated into the brain parenchyma (Fig 1a) The areas with exudation of EBA were strictly confined to the lateral parts of the brain stem and the first and second cercal cord segments No significant exudation was observed within other parts of the CNS In animals sacrificed immediately after the pressure pulse these areas were distinctly demarcated and multifocal and later became confluent The vascular leakage diminished considerably ithin 15 minutes after the pressure pulse The exudation of EBA was more extensive as the amplitude of the pressure pulse was increased The concussive reaction as judged by the blood pressure rise bradycardia apnea and loss of reflexes for a period generally used as indi ative of experimental brain concussion (*Denny Brown* 1945) al o was mo e severe with increasing pressure amplitude and the animals died within a few minutes following pressure pulses exceeding ? 5 atm peak amplitude

In the areas with an increased vascular permeability EBA coul l also be traced to neurons and glial cells (Fig 1 b)

Discussion

The uptake in walls of blood vessels and penetration outside the vessel following the brief pressure pulse was strictly confined to the lateral parts of the br in stem and the upper parts of the cervical cord It may be that the deformations of the brain tissue are of high magnitudes within these parts in this type of injury In similar animal experiments (*Lindgren & Rinder* 1966) and also in cases of blo vs to intact cadaver skulls (*Lindgren* 1966) m ked pressure differences occur in the vi i nity of the foramen magnum and pressure differences are considered to produce she r deformations in these regions (*Gurdjian & Lissner* 1961)

The labelled albumin had extended into the brain parenchyma in animals sacrificed within 30 seconds after the pressure pul e Therefore a direct mechanical lesion of the vascular system of certain parts of the CNS may be a factor of importance *for the development of brain oedema in head injuries*

Diffuse cytopla mi and nuclear FBA fluorescense was often seen within neurons and glial cells in areas with exudation of the labelled albumin Similar phenomena ha e been observed in brain oedema induced by cold lesions and by the injection of mercury compounds they have been considered to denote injury of the cells (*Steinwall & Klat o* 1966 *Hamberge & Hamberger* 1966)

Summa y

Increa ed vascular pe meabilit with exudation of labelled albumin has been observed in experimental brain concus ion n rabbits induced by a pressure pulse of a few milliseconds duration within the skull The exudation was confined to the lateral parts of the brain stem and the upper cervical cord No significant exudation was observed in other parts of the CNS

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EFFECT OF 3,4 BENZPYRENE ON THE DEVELOPMENT OF THE CONTACT ALLERGY TO 2,4 DINITROCHLOROBENZENE IN GUINEA PIGS

By

GUNNAR V ALM

Received 11 v 67

Carcinogenic polycyclic hydrocarbons have been shown to depress the humoral antibody response (*Malmgren et al* 1952 *Davidsohn et al* 1956 *Stjernsward* 1965 1966 a b *Ball et al* 1966) and impair the homograft rejection (*Rubin* 1960 1964 *Stjernsward* 1965 *Linder* 1962 *Ball et al* 1966)

It was suggested by *Prehn & Main* (1957) that the impairment of the immune response may be related to the carcinogenic activity of 3 methylcholantrene

There have been no previous investigations on the effect of carcinogens on delayed type hypersensitivity reactions like the contact allergy to simple chemicals Such immunological reactions may be classified as cellular immunity together with homograft immunity (*e g Chase* 1965)

The present investigation was therefore undertaken to study the effects of 3 4 benzpyrene on the development of contact allergy to the simple chemical 2 4 dinitrochlorobenzene in guinea pigs as measured by the allergic skin response

MATERIAL AND METHODS

Animals 20 female albino guinea pigs each weighing approximately 300 grams at the beginning of the experiment

Chemicals 3 4 benzpyrene (Sigma) was administered as a 1 ?5 per cent solution in equal parts of acetone and olive oil ?5 μl was applied on the left shoulder of the guinea pigs Control animals were given the acetone olive oil solvent only

2 4-dinitrochlorobenzene was utilized as the contact allergen The guinea pigs were sensitized with ?5 μl of a 0 per cent solution in acetone applied adjacent to the 3 4 benzpyrene treated area on two consecutive days The animals were skin tested by the topical application of 10 μl of a 0 per cent solution of DNCB in ethanol on a ? cm^2 area on the right shoulder

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The work was supported by the *Swedish Medical Research Council* and the *Faculty of Medicine University of Uppsala*

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TABLE

DNCB test Readings in DNCB Sensitized Animals
Group B = Short Term 3 4 Benzpyrene Treatment

Time after testing (hours)	Groups and animal number A									
	1	2	3	4	5	6	7	8	9	10
4	++	+	+	+	+	+	±	+	±	++
12	+++	+++	++	+++	+++	+++	++	+++	+++	+++
24	+++	+++	+	+++	+++	++	+++	+++	+++	+++
48	++	+++	+	+++	+++	+	+	++	+++	+++

Design of the experiment Three groups were included in the experiment

A 10 animals were treated with 3 4 benzpyrene every other day for 14 days and on days 14 and 15 immunized with DNCB

B 5 animals were treated according to the same scheme with only acetone olive oil and with 3 4 benzpyrene on day 14 and subsequently immunized on days 14 and 15 with DNCB

C 5 animals were treated with acetone olive oil for 14 days according to the same scheme as group B and then immunized with DNCB on days 14 and 15

Recording of the tests The skin tests were read at 0 4 12 24 and 48 hours Visual observation with grading of the test reactions with reference to the degree of erythema and induration from — to +++ was performed according to *Groth (1964)* ± indicates uncertain reactions The thickness of a fold of the skin in the test area was measured by means of skin calipers (Schnelltaster System Kroplin Type ... 02 T H C Kroplin GmbH Schluchtern Hessen Germany)

At 48 hours the skin tests were excised and 5 μ paraffin sections were stained with Mayer s haematoxylin-eosin The number of lymphocytes infiltrating the epiderm was estimated utilizing the method devised by *Groth* (1964) A total of 400 cells was counted in the epidermis of the test area

RESULTS

All guinea pigs sensitized with DNCB exhibited strong *allergic reactions* when tested by epicutaneous application of the allergen 7 days after sensitization

Naked eye inspection and palpation (Table 1) Groups A and C exhibited reactions with a peak at 12 hours this is most clearly seen in Group C Group B revealed a somewhat different pattern as the allergic reactions were generally more intense at 24 hours and tended to persist until the excision of the tests at 48 hours

Skin thickness in test areas Table 2 a gives the percentage increase in skin thickness The mean values of the B group are significantly higher than those of groups A and C from 12 hours on No differences *between groups* A and C were noted (Table 2 b)

Epidermal infiltration of lymphocytes into the test areas The percentage of lymphocytes in the epidermis in the central parts of the test areas at *48 hours* are given in Table 3 a Groups A and B showed both a significantly larger number of infiltrating (small) lymphocytes than Group C No difference between Groups A and B could be detected (*Table 3 b*)

1
Group A = Long Term 3 4 Ben pyrene Treatment
Group C = Untreated Controls

Groups and animal number										Time after testing (hours)
		B					C			
11	12	13	14	15	16	17	18	19	20	
++	++	++	+	+	+	+	+	±	+	4
+++	+++	+++	+++	+++	+++	+++	+++	++	+++	12
+++	+++	+++	+++	+++	+++	++	+++	+	++	24
+++	+++	+++	++	+++	+	++	+	+	+	48

DISCUSSION

The present investigation demonstrates that 3 4 benzpyrene in comparatively small amounts administered during a short time enhances the inflammatory reaction in contact allergy to the simple chemical 2 4 dinitro 1 chlorobenzene The stimulatory effect was recorded as an increased oedematous reaction in the test areas and an increased epidermal infiltration of small lymphocytes

TABLE 2 a

Percentage Increase in Skin Thickness in the DNCB Test Area
Mean ± Standard Error

Group	No of animals	Percentage increase in skin thickness time after skin test application (hours)			
		4	12	24	48
Long term treated (A)	10	7 6 ± 1 9	37 4 ± 4 1	3?2 ± 4 7	3?7 ± 4
Short term treated (B)	5	11 8 ± 3 9	55 ? ± 3 3	47 8 ± 3 3	60 6 ± 4
Controls (C)	5	3 6 ± 2 9	30 4 ± 7 5	27 6 ± 7 8	26 8 ± 2

TABLE 2 b

Statistical Evaluation of the Results Given in Table 2 a The Student s t-test W
Used Group A Long Term 3 4 Ben pyrene Treatment Group B Short Term
3 4 Ben pyrene Treatment Group C Untreated Controls

Tested groups	Time after skin test application (hours)			
	4	12	24	48
A - B	t = 1 07 0 05 < P	t = 2 79 P < 0 01	t = 2 13 P < 0 05	t = 3 P < 0 0
A - C	t = 1 14 0 05 < P	t = 0 94 0 05 < P	t = 0 52 0 05 < P	t = 0 0 05 <
B - C	t = 1 69 0 05 < P	t = 3 03 P < 0 05	t = 2 38 P < 0 05	t = 6 P < 0

TABLE 3 a

Percentage Epidermal Lymphocyte Infiltration in 48 Hour DNCB Skin Tests Means of the Groups ± Standard Error

Group	No of animals	Infiltration mean ± SE (%)
Long term treated (A)	10	6 53 ± 0 36
Short term treated (B)	5	7 15 ± 0 19
Controls (C)	5	7 95 ± 0 32

TABLE 3 b

Statistical Evaluation of the Results Given in Table 3 a The Student's t-test Was Used

Tested groups	t = values and probability	
A – B	t ≈ 3 07	P < 0 05
A – C	t ≈ 6 75	P < 0 001
B – C	t ≈ 11.39	P < 0 001

A larger dose of 3 4 benzpyrene administered during a longer period caused *only an increased number of infiltrating epidermal small* lymphocytes

The carcinogen 3 4 benzpyrene can influence the outcome of the allergic test reactions in at least two ways

First it can act on the induction of the allergic state *Second* it can affect the inflammatory response in a more or less nonspecific way

As to the possibility of an effect on the induction of the allergy it is known from earlier works by *Stjernsward* (1965 1966 b) that poly cyclic hydrocarbon carcinogens like 3 4 benzpyrene suppress the immune response in mice to erythrocytes as measured by the plaque technique of *Jerne* (*Jerne & Nordin* 1963) *Malmgren et al* (1952) and *Davidsohn et al* (1956) demonstrated that 3 methylcholantrene depres sed the humoral antibody response and *Malmgren et al* in addition showed that other carcinogens but not their noncarcinogenic analogues depressed the immune response Further *Ball et al* (1966) registered a long lasting depression of the antibody response after injection of 9 10-dimethyl 1 2 benzanthracene into newborn mice *Linder* (1962) and *Stjernsward* (1965) showed a prolonged homograft survival in methylcholantrenetreated mice however only after the tumor had ap peared Thus it is obvious that carcinogenic polycyclic hydrocarbon can depress immune responses In the present investigation a stimula tion of an immune reaction was noted *Rubin* (1964) observed a sti mulating effect of the carcinogen 10 methylcholantrene on lymphoid tissue and a prolonged homograft survival in the carcinogentreated animals He postulated an adjuvant effect of the carcinogen with stimu lation of the synthesis of antibodies that caused an enhancement of

tumour growth If an adjuvant action of 3 4 benzpyrene is anticipated this might then divert or enhance the immunogenic stimulus of the DNCB

Adjuvants like Freunds adjuvant are since long known selectively to enhance delayed type allergy of which the contact allergy to DNCB is an example It is also known that many other substances can enhance the immune response *e g* endotoxins (*Kind & Johnson* 1959) and antimetabolites like 5 fluoro 2 deoxyuridine (*Merritt & Johnson* 1963) when administered under certain conditions Of special interest with regard to the present communication is the recent report that the fluoropyrimidines 5 fluorouracil and 5 fluoro 2 deoxyuridine enhanced the existing delayed hypersensitivity in treated cancer patients (*Blomgren et al* 1965) There are also many other examples indicating that adjuvants can cause qualitative differences in the immune response (*White et al* 1963 *Benacerraf et al* 1963 *Coe* 1966)

The action of the carcinogen can conceivably also be direct on the inflammatory process It has been shown that 3 4 benzpyrene is accumulated intracellularly in the lysosomes and the Golgi apparatus (*Allison & Young* 1964) of cells where it increases the permeability of the cell organelles Lysosomes and lysosomal enzymes have been implied as mediators of the inflammatory response (*Weissman* 1965) 3 4 benzpyrene might therefore enhance the inflammatory response by facilitating the release of hydrolytic enzymes or other inflammatory agents from the lysosomes

Vitamin A which has similar effects as 3 4 benzpyrene on the lysosomes of cells (*Allison & Young* 1964) has been shown to induce the rejection of both autografts and homografts in the rabbit (*Nelken et al* 1965) perhaps by activating proteolytic enzymes (*Picl et al* 1963) at the site of graft rejection *Uhr et al* (1963) have shown that also the delayed hypersensitivity response is inhibited by pronounced experimentally induced A hypervitaminosis This inhibiting effect may be explained by the results obtained by *Dingle* (1961) *Janoff & McCluskey* (1962) and *Weissman & Uhr* (1963) which indicate that large doses of vitamin A depletes lysosomes of acid phosphatases and other lysosomal enzymes Conceivably this would diminish the *in vivo* reactivity of cells while small doses only labilize the cells and thus increase the reactivity A similar mechanism would explain the discrepancy between the effects of small and large doses of 3 4 benzpyrene on the skin reactivity observed in the present investigation

Any explanation of the apparent increase of infiltrating epidermal lymphocytes *both* in long term and short term 3 4 benzpyrene treated animals can not be found

Further investigations using also non-carcinogenic hydrocarbons are necessary if a choice is to be made between the discussed alternatives relative to the action of 3 4 benzpyrene on the contact allergy to DNCB

SUMMARY

The influence of 3 4 benzpyrene on the development of contact allergy to 2 4 dinitrochlorobenzene has been studied in adult female albino pigs The following observations were made

1 Long term treatment (14 days) increased the allergic response as measured by the epidermal infiltration of small lymphocytes but did not affect the degree of oedema

2 Short term treatment (2 days) at the time of sensitization increased the epidermal infiltration of small lymphocytes as well as the degree of oedema

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MALES WITH XO Ph^1 POSITIVE CELLS A CYTOGENETIC AND CLINICAL SUBGROUP OF CHRONIC MYELOGENOUS LEUKAEMIA?

Report of a Case

By

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Received 4 xii 67

Since 1962 a number of male patients with *chronic myelogenous leukaemia* have been described in whom the bone marrow and peripheral blood contained Ph^1 positive cells with 45 chromosomes lacking a small acrocentric member In one case it was not possible to decide whether the Y or a (21 22) member was lacking as the Y chromosome greatly resembled the small acrocentric autosomes (*Engel et al* 1965) In another case the absent chromosome was probably Y (*Atkin & Taylor* 1962) In their patient *Grouchy et al* (1966) were able to decide that Y was absent in the majority of the cells In the remaining 5 patients (*Tough et al* 1963 *Speed & Lawler* 1964 *Lawler & Galton* 1966) it seemed to be possible to identify the absent chromosome as the Y

In 7 out of these 8 patients lymphocytes and or skin fibroblasts were found to have normal karyotypes (*Atkin & Taylor* 1962 *Tough et al* 1963 *Speed & Lawler* 1964 *Engel et al* 1965 *Grouchy et al* 1966)

Tough et al (1963) suggested that patients with XO Ph^1 positive cells belonged in a cytogenetically defined subgroup of chronic myelogenous leukaemia *Speed & Lawler* (1964) observed very protracted courses of disease in 2 patients with such cells and stressed the possibility that the protracted character of the disease in these patients was due to the XO Ph^1 positive karyotype *Grouchy et al* (1966) were able to confirm this suggestion and pointed out that their patient had *no children* as was the case with 2 patients published by *Atkin & Taylor* (1962) and *Engel et al* (1965)

These observations seem to outline a *subgroup of chronic myelogenous leukaemia* consisting of males with XO Ph^1 positive cells and

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frequently characterized by childlessness and protracted chronic courses of disease

It is intended with the present paper to describe a patient with chronic myelogenous leukaemia who seems to belong in this group The patients is a male aged 49 years He is married but has no children After 4 years of chronic myelogenous leukaemia he is still in the chronic phase of the disease In the initial stage of the disease his Ph¹ positive blood culture cells showed XO/XY mosaicism

CASE HISTORY

A male aged 45 years was admitted in July 1963 on account of extreme fatigue after 2 febrile periods 3 months and 1 month earlier On the whole his previous health had been good He looked moderately anaemic The sex characters were normally developed *The spleen was found to reach the transverse umbilical plane* The blood status appears from Table 1 The bone marrow was hyperplastic with a violent displacement of myelopoiesis to the left and scanty erythropoiesis During treatment with busulphan good clinical and haematological remission was obtained in the course of a few weeks During continuous treatment with small doses of busulphan (1–2 mg a day) the condition remained stable with 5 000–30 000 leucocytes per mm³ no myeloblasts and 0–5 per cent promyelocytes in the peripheral blood and without palpable spleno or hepatomegaly The haematological status on May 8th 1967 appears from Table 1

Cytogenetic investigations of blood cultures were performed 2 weeks 8 weeks and nearly 4 years after establishment of the diagnosis Times of sampling and relevant *haematological data are given in Table 1 Whereas the first 2 cultures showed lively* proliferation very few mitoses were found in the last culture (see Table 2) No bone marrow samples were obtained from the patient

TABLE 1

Haematological Data from the Time of Diagnosis and the Times of Sampling for Chromosome Investigation

	Before treatment	July 13th 1963	August 26th 1963	May 8th 1967
Haemoglobin (g/100 ml)	10 5	10 4	13 1	15 1
Leucocytes (× 10³/mm³)	117 9	104 0	45 6	7 2
Thrombocytes (× 10³/mm³)	291	430	35[illegible]	243
Myeloblasts (%)	2	4	—	—
Promyelocytes (%)	—	4	—	—
Myelocytes (%)	12	19	16	5
Metamyelocytes (%)	12	12	6	2

TABLE 2

Distribution of Ph in Analysed Blood Culture Cells

Blood culture	Analysed cells			
	Ph¹ positive	Ph negative	Ph¹	Total
July 13th 1963	40	2	2	50
August 6th 1963	21	22	3	40
May 8th 1967	—	2	—	2
Total	67	26	5	98

TABLE 3

Karyotypes of Ph¹ Positive Cells from Two Blood Cultures

Blood culture	Chromosome number	Karyotypes: Absent chromosomes				Extra chromosomes	Number of cells
July 13th 1963	46						27
	45				Y		19
	45		(19–20)				1
	44			(21–22)	Y		1
	44		(19–20)		Y		1
	44	(6–X–12)			Y		1
	42	(6–X–12)	(19–20)	(21–22)	Y		1
	Total						46
August 26th 1963	46						10
	45				Y		8
	44			(17–18)	Y		1
	45		(13–15)				1
	47					(6–X–12)	1
	Total						1

METHODS

The blood samples were cultured for 48 hours in the presence of phytohaemagglutinin in accordance with a slight modification of the technique described by Moorhead *et al* (1960). The slides were air dried and Giemsa stained. Mitoses were analysed directly under the microscope irrespective of presence or absence of Ph¹. The numbers of analysed cells appear from Table 2.

RESULTS

Table 2 shows the distribution of Ph¹ in the analysed cells and Table 3 the karyotypes of the Ph¹ positive cells from the first 2 cultures. Both cultures contained diploid Ph¹ positive (*Fig. 1*) and *XO Ph¹ positive* cells (Fig 2) in roughly equal proportions. The Ph¹ prevalence decreased from 95 8 per cent in the culture established on 13 7 1963 to 48 8 per cent in the culture from 26 8 1963. The decrease was not accompanied by changes in the relative proportions of diploid Ph¹ positive cells (47 8 per cent and 47 6 respectively of the total Ph¹ positive cells of each culture) and XO Ph¹ positive cells (41 3 per cent and 38 1 per cent respectively of the total Ph¹ positive cells of each culture).

The culture from 8 5 1967 yielded no analysable Ph¹ positive cells and only 2 Ph¹ negative cells. The karyotypes of the latter were normal.

A total of 26 Ph¹ negative cells were analysed (Table 2). Three out of these lacked a small acrocentric chromosome, a (21–22) member in 2 and the Y chromosome in 1 cell.

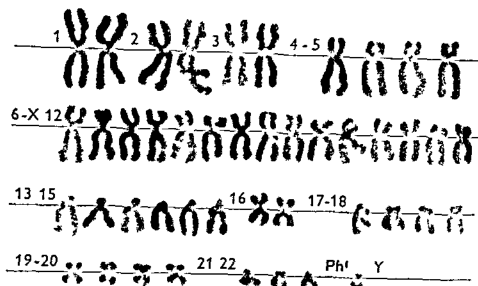


Fig 2
45 XO Ph^1 positive karyotype of blood culture metaphase from the same patient as Fig 1

one patient with a 10 year course of disease. Nothing is stated about 2 patients (*Tough et al* 1963) and 1 patient was examined shortly after establishment of the diagnosis of chronic myelogenous leukaemia (*Atkin & Taylor* 1962). The present patient has had the disease for 4 years. The clinical and haematological conditions have been extremely stable and good without any sign of incipient acute transformation of the disease.

These observations seem to indicate a causal connection in some patients between the cytogenetic picture on one hand and low fertility and a protracted chronic course of disease on the other.

In all 6 cases where it was investigated lymphocytes and/or skin fibroblasts were found to contain the Y chromosome. In 25 out of the 26 Ph^1 negative blood culture cells from the present patient the Y was observed. Probably absence of Y in the remaining cell is due to artificial chromosome loss.

Absence of Y seems to be restricted to the cells produced in the bone marrow. On this background it is very difficult to explain the apparently low fertility of certain patients with XO Ph^1 positive cells. Even the assumption of frequent losses of Y in the gonadal cells cannot explain the childlessness. On the assumption of an abnormally high tendency to non disjunction divisions in these patients a low fertility

might be explained but the protracted chronic course of disease without any sign of cytogenetic evolution in the Ph[1] positive cells observed in an essential proportion of the patients indicates a low incidence of abnormal cell divisions in the bone marrow Apparently there are no observations suggesting low fertility in patients with other aneuploid Ph[1] positive clones

It must be stressed however that there is no conclusive evidence of low fertility in the patients in question Including the case reported by *Engel et al* (1965) 4 patients are apparently childless and 1 has 4 sons Two out of the 4 childless patients were unmarried and nothing is known concerning the fertility of the female partner in the other 2 cases Finally the patient material is so small that it is impossible to exclude that the occurrence of 4 childless patients is accidental

It is generally accepted that the Y chromosome contains few genes besides the sex determining ones If this is the case XO Ph[1] positive and XY Ph[1] positive diploid cells have probably similar cellular phenotypes and must be expected to respond similarly to the selectional pressure of the leukaemic organism If the XO Ph[1] positive line has developed from the diploid Ph[1] positive line it must however in most cases have outgrown the original diploid cells and must therefore probably possess a relative advantage But acting during many cell generations even a very modest relative advantage may lead to this result

It has been shown that aneuploid Ph[1] positive clones develop primarily in the terminal stage of chronic myelogenous leukaemia and that they are characterized by accumulation of supernumerary chromosomes (*Pedersen* 1966b) Additionally hyperdiploid Ph[1] positive cells seem to originate from the most primitive myeloid cells (*Pedersen* 1966a) These observations indicate that cytogenetic evolution in the Ph[1] positive cell population may be responsible for clinical and haematological exacerbation of the disease If diploid Ph[1] positive and XO Ph[1] positive cells have much the same capacity of proliferation and survival in leukaemic organisms the clinical and haematological stability of the patients is likely to be due to absence of cytogenetic evolution in the Ph[1] positive cell population rather than to the XO Ph[1] positive karyotype *eo ipso* This assumption is in accordance with a case described by *Lawler & Galton* (1966) (Patient D C) After a chronic phase lasting 7½ years the disease underwent an acute transformation At the same time the 45 chromosome XO Ph positive cell population of the chronic phase was replaced by a hyperdiploid XO Ph[1] positive population

SUMMARY

A number of patients with chronic myelogenous leukaemia have been described in the literature in whom blood culture and/or bone marrow cells have exclusively or predominantly XO Ph positive karyotypes All of them are males The majority of cases from which relevant in

formation is available are characteristic in that the patients concerned are childless and have protracted chronic courses of disease In the present report a 49 year old male is presented whose Ph¹ positive blood culture cells consist of roughly equal proportions of 46 XY and 45 XO cells He is married but has no children He has a 4 year chronic course of disease behind him but absence of clinical or haematological signs of acute transformation of the disease

It seems to be impossible to deny but very difficult to explain a causal connection between the cytogenetic picture and the apparently low fertility of these patients In most cases acute transformation of the disease is preceded by cytogenetic evolution leading to establishment of hyperdiploid Ph¹ positive clones If XO Ph¹ positive and XY Ph¹ positive cells are assumed to have similar cellular phenotypes and consequently to have roughly equal capacities of survival and proliferation in the leukaemic organism the protracted chronic courses of disease in many of these patients are probably not due to the XO Ph¹ positive karyotype *eo ipso* but a consequence of absence of cytogenetic evolution in their Ph¹ positive cell populations

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ULTRASTRUCTURAL FEATURES OF CROOKE'S CHANGES IN PITUITARY BASOPHIL CELLS

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Received 16 vii 67

Patients with Cushing s disease have changes in the pituitary basophil cells originally described by Crooke in 1935 The nature of these changes involving the nucleus as well as the cytoplasm has been debated (5 7 11) nowadays they are generally accepted as degenerative changes secondary to overload of corticosteroids (16) Since their ultrastructural features have not been described we are presenting electron micrographs from a patient with typical Crooke s changes in the pituitary basophils

MATERIAL AND METHODS

The patient was a 29 year old woman with advanced signs of Cushing syndrome An expansive process could be demonstrated radiologically in the sella region and since laboratory tests indicated hypophyseal overactivity a transsphenoidal hypophysectom was performed At operation material was taken for histological examination (formalin fixation and paraffin embedding) and for electrone microscopy The latter material was fixed in osmium tetroxide (14) embedded in *Epon* (10) and cut with an LKB Ultrotome Section staining with uranyl acetate and lead acetate (15) increased the contrast To achieve the best comparison between light and electron microscopy thick Epon sections were sefained with PAS and Sudan black.

RESULTS

Light Microscopy

The tissue obtained at the operation consisted only of the peripheral parts of the pituitary surrounding the tumour All cells types of the gland were present *i e* eosinophils basophils and chromophobes The basophil cells were more numerous than normal and the majority of them had obvious changes corresponding to Crooke s changes the cytoplasm was partly hyalinized and vacuolated the nucleus enlarged and in more advanced phases irregular in shape

Electron Microscopy

On the whole the cytoplasmic changes dominate the picture while the nuclear changes seem to be subordinated

Cytoplasmic ground substance A fibrillar mass of low electron den



Fig 1
Moderately granulated basophilic cell with partly disintegrated secretory granule accumulated near the nucleus (× 5 000)

sity appears in the cytoplasmic ground substance It is most prominent in areas devoid of cell organelles In the less severely affected cells the fibrillar mass occupies the peripheral zone and surrounds secretory granuls and other organelles nearer the nucleus (Fig 1) Sometimes however the empty fibrillar zone which corresponds to the light microscopic hyalinization is located centrally The most severely affected cells had this empty fibrillar change throughout the cytoplasm (Fig 3)

The individual fibrils are thin and measure 40–80 Å in diameter They may run singly but are more often arranged in irregular bundles The orientation of these varies and no special pattern can be demonstrated

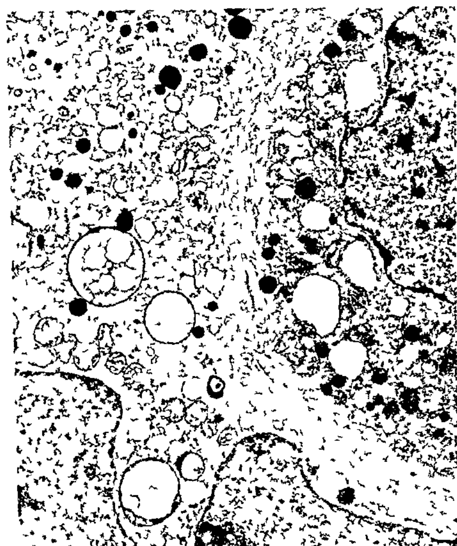


Fig ⁹
Crooke s cell upper right with fibrillar zone al)n th t r I le \ t th b ence of a pla ma membrane The cytoplasm of th n ghl t r l 1 l ge bo lies which apt ear to represent PAS po itive gr n le l t tı n (× 15 000)

Cell membrane With few exceptions n c l m nbr me ould be de monstrated around the Crooks cells to l a h c t p l m in direct continuity with that of the surroundin c ls (Fig 2) The pheno-menon cannot be due to an artefact since cell b un laries are present between the other cellular components of the specim n

Endoplasmic reticulum The endopl ısmic reticulum is sparse in cells



Fig 3
Crooke's cell with the fibrillar change involving almost the whole cytoplasmic area Note the lipid body near the nucleus (× 5 000)

containing the fibrillar material Some dilated cisternae belonging both to the smooth and rough surfaced types persist even in greatly altered cells

Ribosomes In addition to ribosomes attached to the endoplasmic reticulum a few lay free in the cytoplasm singly or in rosette formation (polysomes) even in areas with the fibrillar change

Secretory granules The number of secretory granules varies greatly They are numerous in many of the less altered cells but sparse in the most affected ones The distribution of granules followed different patterns but they were usually concentrated near the nucleus

The granules are strongly PAS positive in the thick Epon sections They have a mean diameter of 0.4 μ (range 0.35 0.4 μ) When intact

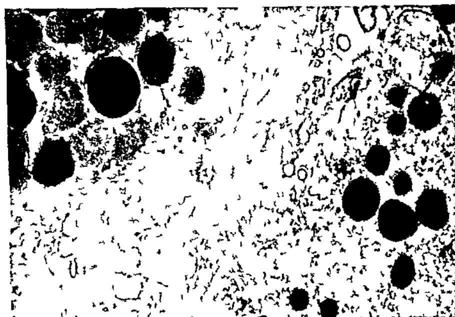


Fig 4

Secretory granules some of which appear to be disintegrating The fibrillar material is seen between the two groups of granules (× 47 500)

they are strongly electron dense and surrounded by a membrane In the altered cells however their outlines are less distinct and their density diminished (Fig 4)

Mitochondria There is some swelling and rupture of the mitochondrial cristae possibly due to the fixation

Other cytoplasmic bodies In addition to the well known organelles described above the cytoplasm of Crooke s cells contains bodies of two main types with many intermediate forms One type is rounded or slightly elliptic and has considerable density (Fig 3) The size varies but often the diameter of the bodies reaches several micra They are often located near the nucleus The bigger bodies in particular show irregular holes and have the morphology of lipid particles In thick Epon sections the e bodies stain with Sudan bl ck

The other type of cytoplasmic body is al o relatively l [illegible] p [illegible] some micr i at least in one dimension) The ele tron d n ty [illegible] than that of the cytoplasm but definitively less than [illegible] the fat particles and of the secretory granules (Fig) As n [illegible] type of body there may be holes in the matrix Th bodi ar [illegible] PAS positive in thick Epon sections

Bodies of combined type (Sudan black and PAS positive material) exist especially as a PAS positive envelope surrounding a sudanophilic globule

Nucleus The nuclear changes were apparent in paraffin sections The nuclei are enlarged irregular in shape and with a somewhat condensed chromatine material evenly distributed throughout the nucleus Nucleoli may be present

DISCUSSION

In 1935 when *Crooke* described the changes in the basophil pituitary cells from patients with Cushing s syndrome the disease was generally considered to be caused by hormone overproduction from a basophilic *adenoma or from the pituitary gland itself* and the alterations were regarded as signs of hyperactivity It was later shown that the majority of Cushing patients had a primary overactivity of their adrenal glands Thus a possibility existed that the Crooke lesions might be of a degenerative nature From cytological and cytochemical analyses *Wilton Thorell & Sundwall (1954)* also obtained evidence for this It is also known that similar changes can be induced in pituitary glands of animals after cortisone administration (6)

The microscopical appearance of the basophil cells in our case fits well with the description in *Crooke* s original article (3) The granules in the cells are considerably larger than secretory granules observed in basophil cells of rats (8) However large granules in normal human glands and in rat pituitaries under pathological conditions for example after castration have been described

The dominating change seen with the light microscope is hyalinization *i e* the conversion of cytoplasmic areas to glassy amorphous parts with moderate eosinophilia This phenomenon is a common finding in pathological material both intra and extracellularly In the former position the hyaline is generally regarded as a degenerative sign Chemically the hyaline material probably represents protein

In the electron microscope the hyaline material shows different morphological patterns In studies of hyaline degeneration of rat liver cells *Bruni* (2) demonstrated that the material was composed of vesicles and tubules belonging to the endoplasmic reticulum In other studies the hyaline was found to consist of amorphous finely granular or fibrillar material (4) In the cells investigated ultramicroscopically by us the hyalinized areas have a fibrillar structure and usually lack cytoplasmic organelles They show no similarity to the endoplasmic reticulum It is interesting to note that Crooke s cells usually are devoid of limiting cell membranes and that an extensive fibrillar zone here appears to constitute a boundary

Fine filaments occur normally within different cells for example *tonofibrils in skin epithelium and myofilaments in smooth muscle* In addition to these more well known cases several authors have described fine filaments in a wide variety of cell types *De Petris et al* (12) among others concluded that these structures are normal or physio

logical constituents of the cells (not artefacts) though of unknown function It is not possible to ascert un whether these filaments arise by conversion of cytoplasmic proteins by an active synthetic process or in some other way

SUMMARY

Electron micrographs are presented showing the alterations in Crooke s cells of the pituitary gland Their dominating feature the hyalinization of the cytoplasm corresponds ultramorphologically to a fibrillar mass enclosing only small amounts of cell organelles The origin structure and function of the fibrils is unknown

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STUDIES IN ORAL LEUKOPLAKIAS

14 *Sulphydryl Group and Disulphide Bonds in Normal and Leukoplakic Epithelium before and after Vitamin A Application*

By

F A VON BULOW and G RENSTRUP

Received 19 viii 67

The content and distribution of sulphydryl groups and disulphide bonds in the oral mucosa have been the subject of only a few investigations *Turesky Crowley & Glickman* (1957) studied the occurrence and distribution of sulphydryl groups and disulphide bonds in gingiva They found a greater content of sulphydryl groups in keratinized and parakeratinized layers than in the deeper layers of the epithelium while the disulphide bonds were distributed evenly throughout the epithelium Investigating oral leukoplakic epithelium *Turesky Glickman & Provost* (1961) found the greatest content of sulphydryl groups in the granular layer of orthokeratinized epithelium In the stratum corneum the content of sulphydryl groups was lower than in the granular layer and in the Malpighian layer it was the lowest In parakeratotic epithelium the greatest concentration of sulphydryl groups occurred in stratum corneum The greatest concentration of disulphide bonds in orthokeratotic lesions was found in stratum corneum whereas parakeratotic lesions were negative In gingival epithelium *Toto & Grandel* (1966) found that the keratinized layers had a greater content of sulphydryl groups than the remaining epithelial layers They also found that the disulphide bonds were more frequent in stratum corneum and stratum granulosum than in stratum spinosum and stratum basale The authors relate the increased content of sulphydryl groups in the keratinized layers to an increase in density of tonofibrils in these layers *Cohen* (1967) found a discrepancy in the content of disulphide bonds in gingiva using the DDD method by *Barrnett & Seligman* (1952) and using the fluorochrome method by *Jarret et al* (1959) With the DDD method the disulphide bonds were found

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throughout the epithelium with the highest reaction intensity in the horny layers of hyperorthokeratotic and hyperparakeratotic epithelium while the fluorochrome method did not reveal any disulphide bonds. In the normal human buccal mucosa histologically showing an unkeratinized or slightly parakeratinized epithelium *Bülow* (1966) found that the content of sulphydryl groups was negligible in the surface layer, low in the stratum spinosum and a little higher in the basal layer; disulphide bonds were demonstrated with an evenly low intensity in all epithelial layers.

The purpose of this investigation was 1) to compare the content and distribution of sulphydryl groups and disulphide bonds in the epithelium of oral leukoplakias with different keratinization types, 2) to compare these findings with those of normal unkeratinized oral mucosa and 3) to study if Vitamin A had an effect upon the correlation between the content and distribution of sulphydryl groups and disulphide bonds and the type of keratinization.

MATERIAL AND METHODS

The same fifteen patients with oral leukoplakias as used in previous investigations (*Renstrup et al* 1965, *Bülow & Renstrup* 1966) were studied. The age varied from 37 to 74 years and the sex distribution was ten men and five women. The leukoplakias were localized to the cheek and commissural area in eleven cases and to the inferior surface of the tongue in four cases.

Vitamin A was given as described previously (*Silverman et al* 1963 a, b). Ten troches of 75 000 unit Vitamin A acetate per day for two to three weeks were prescribed and the patients were instructed to place a troche until dissolved against the lesion to be studied.

Biopsies were taken from the leukoplakic lesions and from the adjacent clinically normal mucosa of all patients before as well as after Vitamin A application.

After a local infiltration anaesthesia (lidocain 2 per cent) was established in which care was taken not to inject directly into the site of biopsy, 5 mm punch biopsies were taken. The tissue was fixed in neutral formalin, embedded in paraffin and sectioned at 6 μm.

Sulphydryl groups were demonstrated by the method of *Barrnett & Seligman* (1954) using 2,2'-dihydroxy-6,6'-dinaphthyl disulphide (DDD) and the diazonium salt of 5-chloro-o-anisidine (Fast Red RC). Disulphide bonds were demonstrated with the DDD method after blocking of the sulphydryl groups (*Barka & Anderson* 1963) and reduction of the disulphide bonds into sulphydryl groups (*Barka & Anderson* 1963). As a control served sections stained with the DDD method after blocking of the sulphydryl group. The controls were never completely negative.

RESULTS

Sulphydryl Groups

The stratum corneum of the epithelium characterized by hyperorthokeratosis and hyperparakeratosis with a granular layer gave no reaction for sulphydryl groups. In the stratum granulosum the reaction intensity was very high while it was moderate in the spinous layers and in the basal layer of the epithelium of both types. The stratum corneum of hyperparakeratotic epithelium without a granular layer gave a very low reaction for sulphydryl groups and the remain

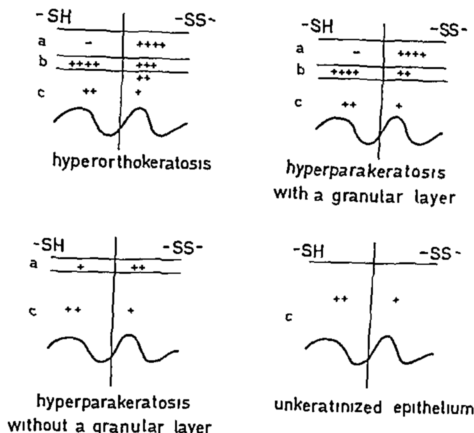


Fig 1

The intensity of staining reaction for sulphydryl groups (—SH) and disulphide bonds (—SS—) in the different layers of four types of keratinization of oral epithelium

— no reaction + ++ +++ and ++++ degrees of intensity of staining reaction a keratin layer b granular layer c spinous layers and basal layer

ing epithelial layers gave a moderate reaction The unkeratinized epithelium gave a moderate reaction in all layers Fig 1

Disulphide Bonds

The reaction for disulphide bonds showed a very high intensity in stratum corneum of hyperorthokeratotic epithelium and of hyperparakeratotic epithelium with a granular layer In the stratum granulosum the reaction was high in hyperorthokeratotic epithelium and moderate in hyperparakeratotic epithelium In the outer spinous layers of hyperorthokeratotic epithelium the reaction for disulphide bonds was moderate while the remaining spinous layers and basal layer gave a low reaction as did all spinous layers and basal layer in the hyperparakeratotic epithelium with a granular layer The stratum corneum of

hyperparakeratotic epithelium without a granular layer showed a moderate reaction and the remaining epithelial layers showed a low reaction as did all the layers in the unkeratinized epithelium Fig 1

DISCUSSION

The results of the present investigation show that the stratum corneum of hyperorthokeratotic epithelium and of hyperparakeratotic epithelium with a granular layer have a very high content of disulphide bonds but contain no sulphydryl groups while the granular layer for these two types of epithelium have a very high content of sulphydryl groups and a moderate one of disulphide bonds This indicates an oxydation in the surface layers of all the sulphydryl groups into disulphide bonds Hyperparakeratotic epithelium without a granular layer contains moderate amounts of disulphide bonds and small amounts of sulphydryl groups in the stratum corneum indicating a lesser degree of oxydation of sulphydryl groups into disulphide bonds

The only difference in content and distribution of disulphide bonds and sulphydryl groups between hyperorthokeratotic epithelium and hyperparakeratotic epithelium with a granular layer is that the oxydation of sulphydryl groups into disulphide bonds seems to start in a lower layer in the epithelium in the hyperorthokeratotic epithelium In the surface layers of hyperparakeratotic epithelium without a granular layer the oxydation of sulphydryl groups into disulphide bonds seems to be incomplete This may indicate that the keratinization process in the hyperparakeratotic epithelium without a granular layer has not been carried through

The sulphydryl rich transitional zone between stratum corneum and stratum granulosum described by *Turesky et al* (1961) in hyperkeratotic epithelium was not observed in the present investigation

Vitamin A is not found to have a direct effect upon the content and distribution of sulphydryl groups and disulphide bonds or upon the content of acid phosphatase (*Renstrup et al* 1965) or upon the content of glycogen (*Bulow & Renstrup* 1966) Thus the way of action of the vitamin is yet unknown

SUMMARY

The occurrence and distribution of sulphydryl groups and disulphide bonds in the epithelium of oral leukoplakic lesions and of normal oral mucosa were studied The results have been correlated with the type of keratinization and with local application of V[illegible] A Sulphydryl groups could not be detected in the strat[illegible]um of hyperorthokeratotic epithelium and of hyperpar[illegible] epithelium with a granular layer in all other epithelial la[illegible] [illegible]ryl groups were present The highest content was fo[illegible] [illegible] layers of leuko

At investigation the normal and vaccinated animals were anaesthetized with 2.5 per cent Nembutal sodium (25-50 mg/kg b.w. i.p.). The peritoneal cavity was opened by an incision between the last two ribs on the left side. One of the veins draining the spleen was incised and blood collected in a heparinized pipette (Heparin® Vitrum, Stockholm, Sweden). Immediately afterwards a splenic artery was incised and arterial blood collected in the same way. The level of anaesthesia was highly important as too deep anaesthesia caused contraction of the splenic arteries. Animals with an ischaemic spleen were not examined.

The blood samples were used for white cell counts in a Burker chamber and for differential counts of white cells in blood smears stained with Giemsa solution. Differentiation was made between lymphocytes, monocytes and neutrophilic, eosinophilic and basophilic granulocytes.

The results in splenic arterial and venous blood were compared in the individual animals, the statistical analysis being based on all the differences in cell content and performed by Student's t test.

In 15 additional males of the same age and weight as stated above the blood from a splenic vein and a splenic artery was compared with respect to the haematocrit value and the number of erythrocytes per mm³ of blood.

RESULTS

Normal Animals

The number of lymphocytes in blood from a splenic vein exceeded that in blood from the splenic artery, the mean difference ± S.E. being 123 ± 57 cells per mm³ ($t = 2.2$, $df = 30$, $p < 0.05$). No difference was present between the number of monocytes and granulocytes (eosinophilic, basophilic and neutrophilic) in afferent and efferent splenic blood. No significant difference was demonstrated between the haematocrit values and the number of erythrocytes of venous and arterial blood from the splenic vessels (Table 1).

TABLE 1

Number of Erythrocytes per mm³ and Haematocrit Values (Per Cent) in Blood from Afferent and Efferent Splenic Vessels. Mean ± S.E.M. Number of Animals within Brackets. The Statistical Comparison Was Based on Paired Differences. No Difference Was Demonstrated.

Source of blood	No. of erythrocytes/mm³ (× 10⁶)	Haematocrit (%)
Splenic vein	5.0 ± 0.2 (7)	42.0 ± 1.3 (8)
Splenic artery	4.9 ± 0.2 (7)	42.6 ± 0.9 (8)
Difference	0.1 ± 0.3 (7)	−0.6 ± 1.1 (8)

Pertussis Vaccinated Animals

A single injection of pertussis vaccine caused lymphocytosis in the blood with a distinct maximum 12 days after the injection, when the number of lymphocytes in splenic arterial and venous blood was increased by 76 and 112 per cent, respectively, as compared with the control values. Vaccination also produced an increase in circulating neutrophilic (pseudo-eosinophilic) granulocytes, which were increased

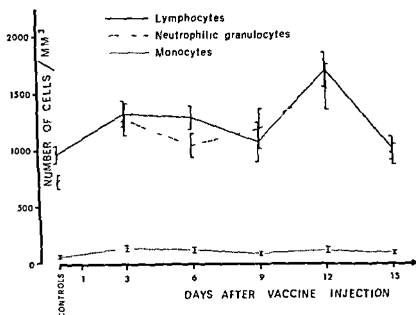


Fig 1

Number of lymphocytes monocytes and granulocytes per mm³ of blood from the splenic artery in normal and pertussis vaccinated guinea pigs (3 6 9 12 and 15 days after a single injection of vaccine) Mean ± SEM

in number as early as 3 days after injection and later increased further to a maximum after 12 days (Fig 1)

At 3 days after antigen injection no significant difference was found between the lymphocyte content of splenic venous and arterial blood whereas at 6 9 and 12 days a larger number was demonstrated in the venous blood ($p < 0.05$ $p < 0.01$ and $p < 0.01$ respectively) The maximal splenic veno arterial difference 602 ± 179 lymphocytes per mm³ of blood was noted 12 days after antigen injection (Fig 2) and was significantly higher than the veno arterial difference in the normal animals ($t = 2.55$ $df = 45$ $p < 0.01$)

The content of other blood cells than lymphocytes did not differ significantly in afferent and efferent splenic blood except 12 days after the vaccine injection The number of monocytes and neutrophilic granulocytes was then higher in the venous blood than in the arterial the difference being significant and almost significant respectively

DISCUSSION

The present investigation disclosed a higher number of lymphocytes in efferent than in afferent splenic blood The haematocrit values and the number of erythrocytes in blood from the splenic vein and the sple

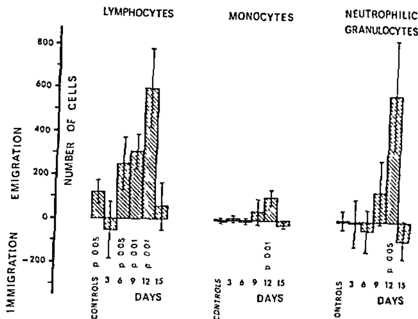


Fig 2

Splenic veno arterial difference in content of lymphocytes monocytes and neutrophilic granulocytes in normal and pertussis vaccinated guinea pigs (3 6 9 12 and 15 days after a single injection of vaccine) An emigration of lymphocytes from the spleen in normal animals and an increased emigration in immunized animals is demonstrated. The statistical analysis is based on the differences in the individual animals Mean difference ± S E M

nic artery were identical The finding indicates proliferation of lymphoid cells in the normal spleen resulting in a greater number of lymphocytes leaving the organ than entering it The existence of an import into the spleen of thymic lymphocytes as postulated by *Fichtelius* (19ɔ3) and *Linna & Stillstrom* (1966) must denote a still greater export of lymphocytes from this organ than indicated by the splenic veno arterial difference in content of lymphocytes observed in the present investigation

The splenic veno arterial *difference in content of lymphocytes in the normal guinea pigs 123 ± 57 cells per mm³ is less than the corre*sponding difference in the thymus 614 ± 157 cells per mm³ (*Ernstrom & Larsson* 1967b) obtained with an identical technique (differential counts of blood smears combined with total white cell count in a *Bürker chamber) However without an evaluation of the blood flow* through the spleen as performed in the thymus (*Larsson* 1966) no direct comparison can be made between the output of lymphocytes from the two organs

In the pertussis vaccinated guinea pigs the splenic veno arterial dif*ference* in number of lymphocytes increased the maximal difference being noted when the lymphocytosis in the blood was most pronounced

Moreover at this time a significantly higher number of monocytes was found in the splenic venous blood than in the arterial The results directly demonstrate a contribution of the spleen to the circulating lymphocytes and monocytes in pertussis vaccinated guinea pigs and are thus in agreement with the observations of *Cannon & Wissler* (1965) indicating that lymphoid cells almost certainly formed in the spleen enter the blood stream during the period of maximal splenic hyperplasia provoked by antigen

Concurrently with the maximal export of lymphocytes from the spleen an emigration of granulocytes was demonstrated This was not the case in normal animals which implies that these granulocytes may either have been produced in the spleen or have accumulated there and been released during immunization

In contrast to the present result in the spleen the same dose of pertussis vaccine produced no significant increase in the venous output of lymphocytes from the thymus (*Ernstrom & Larsson* 1967a) The result favours the view that an increased number of lymphoid cells enter the blood vessels in the spleen after immunization with pertussis vaccine

SUMMARY

The content of different blood cells was compared in afferent and efferent splenic blood of normal and pertussis vaccinated guinea pigs The following results were obtained

1 In normal guinea pigs the content of lymphocytes in efferent splenic blood exceeded that in afferent blood by 123 ± 57 cells per mm^3 No difference was present between the content of other white cells or between the content of erythrocytes

2 In the pertussis vaccinated guinea pigs lymphocytosis was seen in the blood Three days after vaccine injection no significant splenic veno arterial difference in content of lymphocytes was demonstrated whereas at 6 9 and 12 days the difference was greater than in the normal animals The maximal difference 602 ± 179 cells per mm^3 was found at 12 days when the lymphocytosis in the blood was most pronounced Furthermore at this time the efferent splenic blood had a higher content of monocytes and granulocytes than the afferent blood

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TABLE 1

Data on 7 cases of Eosinophilic Granuloma (Reticulo endotheliosis) Diagnosed Cytologically

Case no	Sex	Age at examination	Duration of symptoms (year)	Site	Clinical diagnosis	Histological diagnosis	Treatment	Subsequent course
1	♀	4	0 5	*Occipital bone*	Schüller Christian	*Eosinophilic granuloma*	X rays pitressin steroids	Living 4 years after diagnosis
2	♀	7	0 3	Thyroid gland	Schüller Christian	Eosinophilic granuloma	X rays pitressin steroids Methotrexate	Living 5 years after diagnosis
3	♂	Newborn	0	*Left of neck*	*Letterer Siwe*	*Histiocytosis X*	X rays *steroids*	*Not known*
4	♂	5	0 5	Parietal bone	Schüller Christian	Eosinophilic granuloma	X rays pitressin steroids	Living 2 years after diagnosis
5	♂	24	1	*Clavicle*	Eosinophilic granuloma	Eosinophilic granuloma	X rays	Well 4 years after radiotherapy
6	♂	3	0 3	*Iliac crest*	Schüller Christian	No histologic diagnosis	X rays steroids	Not known
7	♀	67	2	Rib dorsal	Eosinophilic granuloma	Histiocytosis X possible malignant	X rays *after radical operation*	Well 2 years *after operation* and radiotherapy

nosis was established by some other method The series comprised 7 patients In 6 of these patients incisional as well as aspiration biopsy had been performed and the results of the two evaluations could be compared

Five patients were followed up for at least 2 years One patient was a newborn the others ranged in age from 3 to 67 years Two had soft tissue tumours and 5 had skeletal lesions

METHODS

The aspiration technique has been described in detail elsewhere (3 13) A 10 ml Luer Lock syringe furnished with a special one hand grip was used for aspirating the specimen while the palpating hand held the tumour in a suitable position Soft lesions were punctured with a fine (?? gauge) needle and bone lesions with a sternal puncture needle

Smears provided by the aspirates were dried in air The cells were stained by the May Grunwald Giemsa method

RESULTS

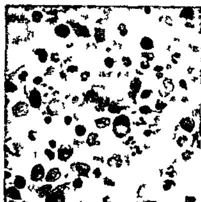
The data for the 7 cases of reticulo endotheliosis are summarized in Table 1 There was full agreement between the diagnosis based on the aspirates and histological sections The features seen at cytological examination of the aspiration biopsy specimens and the picture presented by histological sections from the same lesion are illustrated in Fig 1–6 A shortcoming of puncture cytology is possibly that it does not reveal the partly granulomatous appearance that is seen histologically In none of the patients in whom a diagnosis of reticulo endotheliosis was made on incisional biopsy specimens had the disease been overlooked in the preceding cytological examination of aspirates

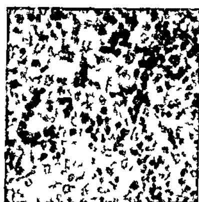
DISCUSSION

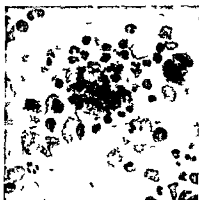
As *Lichtenstein* (10) says there is little if any difficulty in recognizing the histological picture in eosinophilic granuloma provided that there are no artefact alterations that affect the eosinophilia or simultaneous fractures that can result in a picture dominated by the scar tissue formation

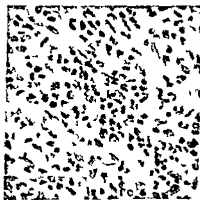
In view of the close agreement between the microscopic appearance presented by the smear from the aspirate and the histological specimens there is little difficulty in obtaining a correct diagnosis of reticulo endotheliosis by aspiration biopsy provided that representative specimens can be obtained as was possible in these 7 cases

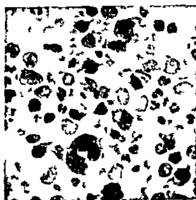
The problems presented by differential diagnosis on smears after aspiration biopsy are the same as those involved in histological sections the fairly common occurrence of mitoses can be a source of misinterpretation with a risk that eosinophilic granuloma may in some cases be taken as sarcoma or malignant lymphoma especially reticulum cell sarcoma or Hodgkin s disease Apart from the presence of mitotic figures there is nothing in the microscopic picture of eosinophilic granuloma that is indicative of malignancy The nuclei are regular stain pale and contain small nucleoles While both Hodgkin s

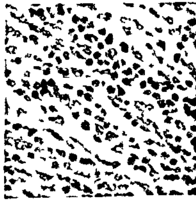

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disease and eosinophilic granuloma display eosinophilia and have cells with two or more nuclei the nuclear abnormalities in Hodgkin s disease are considerably more marked and the enlargement of the nucleoles is more prominent The moderate charges in the nuclei of reticulum cells in eosinophilic granuloma together with the pronounced eosinophilia rule out other diseases such as osteogenic sarcoma or malignant giant cell tumour

Simple bone cysts bone changes resulting from hyperthyroidism reparative giant cell granuloma and giant cell tumours in bone are other possibilities that must be kept in mind but since eosinophilic cells hardly occur in these lesions and since stroma cell components such as fibrocytic or fibroblastic cells are considerably more strongly represented here than in reticulo endotheliosis the differential diagnosis is a fairly simple matter In addition the giant cells that occur in reticulo-endotheliosis are not as prominent as in the previously mentioned lesions and as a rule they do not contain as many nuclei as for instance giant cell tumours

SUMMARY

Seven cases of reticulo endotheliosis are reported in which a diagnosis could be made on the basis of aspiration biopsy examinations In 6 cases the microscopic picture presented by the aspiration specimens was compared with the histological appearances The characteristic features of smears using the above mentioned staining methods are described The differential diagnosis is discussed In this small series the diagnosis of reticuloendotheliosis could be made with an apparently high degree of accuracy on smears from aspiration specimens

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Figs 1–6

Fig 1 Cytological picture presented by the smears of an aspiration specimen of the neck tumour in case 3 A large number of eosinophili granulocytes foam cells reticulum cells with bas phil cytoplasm the one in the middle has 2 nuclei MCG × 500

Fig 2 Histological picture of the tumour in case 3 A large number of eosinophili granulocytes a massive bakground of reticulum cells disper e [illegible] m cell Haematoxylin and eosin × 500

Fig 3 Cytological picture presented by the smear of an a pi at on pe im [illegible] f th clavicle tumour in case 5 A large number of [illegible] philic fa [illegible] ti culum cells eosinophilic granulocytes di per e [illegible] foam cll [illegible]

Fig 4 Histological ection of the tumour in ca 5 N mer [illegible] ell reticulum cells and a few foam cells Note th fil [illegible] llary vessels Haematox lin and eosin × 500

Fig 5 Cytological picture presented by the s nea f an a pi [illegible] men from case 7 Numerous basophilic reticulum cell [illegible] cells and occasional foam cells some multi nu lear [illegible]

Fig 6 Histological picture of the tumour in ca [illegible] r t culum and eo inophilic cells and some fibrocytes [illegible] × 500

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DIAGNOSIS OF GRANULAR CELL MYOBLASTOMA BY FINE NEEDLE ASPIRATION BIOPSY

By

SIXTEN FRANZÉN and BJÖRN STENKVIST

Received 9 1 68

The object of this study was to examine the microscopic picture of granular cell myoblastoma in fine needle biopsy specimens and to study whether the application of this technique would make the tumour identifiable pre operatively without need for incisional biopsy

MATERIAL

The case series consisted of 3 patients in whom fine needle aspiration biopsy examinations had *yielded smears with characteristic features of myoblastoma In all 3* cases the smears were compared with histological sections of the specimens obtained at operation

METHODS

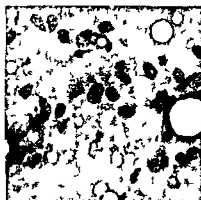
Aspiration technique—The aspiration technique has been described in detail elsewhere (2 8) The cells were aspirated from the tumour by means of a dry air tight 10 ml Record syringe with a Luer Lok connection and a thin (22 gauge) needle The syringe was specially designed to permit one handed manipulation while the palpating hand held the tumour in a suitable position No anaesthesia was necessary

Staining methods—The aspirated cells were spread on a slide with a thick cover glass (Burker chamber)

The slide was then dried in air or fixed immediately in 85 per cent alcohol The air-dried cells were stained by the May Grunwald Giemsa method (MGG) and the alcohol fixed cells by the method of Papanicolaou

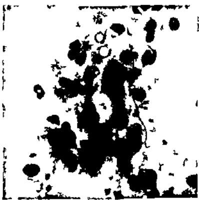
RESULTS

The morbid history of the 3 patients is summarized in Table 1 The similarity between the cytological and histological pictures is illustrated in Figs 1–3 and 4–6 and also in Table 1 which shows the close agreement between the cytological and the histological diagnoses One of the patients (case 3) had a malignant variant of myoblastoma as was evident from the presence of metastases and the high proportion of polymorphous cells observed both cytologically and histologically (Figs 4–6) In none of the cases in which myoblastoma was found at


1


2


3


4


5


6

the histological examination of incisional or surgical specimens had it been overlooked at the previous aspiration biopsy at this department

DISCUSSION

Myoblastoma is usually considered to be myogenic in origin but some workers favour a neurogenic derivation while yet others propose that the tumour arises from fibroblasts or histiocytes (3 4 6 7) Despite this diversity in views on the histogenesis of the tumour there is agreement on the highly specific histological appearance Differential diagnosis poses no real problem since there is no other known tumour that is composed almost exclusively of cells with the typical largely eosinophilic granulation of the cytoplasm displayed by myoblastoma Histiocytes possess a more irregular granulation and are for the most discrete with no cytoplasmic contact between the cells The only tumour that might conceivably be considered as an alternative diagnosis is the specific histiocytic tumour xanthoma but this has a high lipid content and contains crystals that are birefringent in polarized light In addition xanthoma contains a large amount of cholesterol and lipids which stain with Sudan IV None of these characteristics is associated with myoblastoma None of the tumours found in the 3 patients displayed marked nuclear polymorphism in the aspiration specimen from case 3 however a few cells contained bizarre nuclei which suggests malignancy this was also indicated by the fact that the tumour had metastasized

Since 1926 when *Abrikossof* (1) described the peculiar granulated tumour which he called myoma and which differed distinctly from other tumours by virtue of the granulation of the cytoplasm a large

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Figs 1–6

Fig 1 Cytological picture pre ented by an aspiration biopsy specimen from case 2 after air drying and staining by the May Grünwald Giemsa method The cells have pale regular nuclei and a typical greyish violet finely granulated cytpolasm × 500

Fig 2 Aspirate from case 2 after fixation in 95 per cent alcohol and staining by Papanicolaou s metho l As in Fig 1 there are regular nuclei and distinct characteristic granulation of the cytoplasm × 500

Fig 3 Histological appearance of the tumour (m oblastoma) from case 2 Typic l granulation of the cells regular nuclei and sparse fibrocytic st oma Haematoxylin and eosin × 500

Fig 4 Aspirate from case 3 showing the greyish v olet finely granulated t pl m t pical of myoblastoma and a few sl ghtly polymorphous ll nuclei Haematoxylin and eosin × 500

Fig 5 Aspirate from case 3 stained b Papani ol u s method fte f xation in 95 per cent alcohol Here too the typical gr nul ti n is e i nt in some cells larger granules an l some pol morphous c l ru l i × 5

Fig 6 Hist logical picture of excised ne k met st in a In the histological picture too nuclear polymorphism is evident m r le ly than in the benign case in Fig 3 Haematoxylin and eosin × 500

TABLE 1
Three Cases of Granular Cell Myoblastoma Diagnosed by Aspiration Biopsy Sspecimens

Case no	Sex	Age	Location of tumour	Clinical diagnosis	Tumour known to patient (years)	Cytological diagnosis	Histological diagnosis	Treatment	Subsequent course
1	♂	61	Sternal border	Malignant tumour	10	Myoblastoma	Myoblastoma	Radical excision	Well 5 years after operation
	♀	57	Upper arm	Malignant tumour	4	Myoblastoma (Figs 1 and 2)	Myoblastoma (Fig 3)	Radical excision	Well 3 years after operation
3	♀	60	on neck Two swellings	Malignant tumour	6	Myoblastoma probably malig nant variant (Figs 4 and 5)	Myoblastoma Malignant variant (Fig 6)	Radical excision	Operation for primary tumour Metastases in neck 1 year post op Died from coronary infarction 1 year after the second operation No autopsy

number of cases of this type of tumour have been published With a knowledge of the typical gross appearance a histological diagnosis presents little difficulty The possibility of making a diagnosis pre operatively by fine needle aspiration biopsy has not however been reported hitherto

The characteristic microscopic picture presented by myoblastoma in histological specimens is also displayed by specimens obtained by fine needle aspiration biopsy and its recognition suffices for a reliable diagnosis It is also possible that a grading of malignancy of the tumour can be made on the basis of the degree of nuclear polymorphism seen in the aspiration biopsy specimen but as the material contained only one such case no definitive conclusions on this point are permissible

SUMMARY

In 3 cases of myoblastoma a diagnosis could be made pre operatively on the basis of fine needle aspiration biopsy One of these presented clinical signs of malignancy The characteristic picture presented by smears with the staining methods used is shown Because of the typical features of this tumour there is little risk of confusion with any other disease in the microscopic examination of representative specimens

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A CONTRIVANCE FOR BACTERIOCINE TYPING

By

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Bacteriocine typing is based on the ability of bacteria to produce growth inhibiting substances Although it was related to lytic bacterio phage at the time colicine was the first such substance to be observed (4 5 7 8) *Abbott & Shannon* in 1958 (1) described a technique for the demonstration of colicine production of *Shigella sonnei* as an epidemiological marker Colicine typing of *Escherichia coli* has been employed also (6 11)

Potentially bacteriocine typing is a valuable supplement to other methods of typing like phage pattern and serological characterization Sensitivity to the ordinary kind of antibiotics has limited value in this respect The dependence on amino acids (1) and other metabolic pro perties are useful tags for epidemiological surveys

In 1954 *Jacob* (9) described an antibiotic like substance synthesized by *Pseudomonas aeruginosa* pyocine

Wahba et al (3 12) put this into practical use by elaborating a me thod for pyocine typing I have worked with a set of indicator strains selected by this group

The method entailed streaking out each of the strains to be typed diametrically across separate plates Pyocine production took place during over night incubation within a specified range of temperatures After killing with chloroform a set of twelve Pseudomonas strains were streaked perpendicularly to the former The presence of bacterio cine was evidenced by growth inhibition of the indicator strains

Repeated cross streakings of the same indicator strains became necessary (1 6) Carried out one by one this was a tedious and time consuming task

Mechanical devices for multiple simultaneous cross streakings have been constructed (2 10 12) *Lidwell & Carpenter* (10) used a bar from which were suspended nine loops that were moved across the plates simultaneously The *Barrow & Ellis* system (2) transferred the indica tor strains with microscope slides in a special wooden holder and blot ting paper strips soaked with the strains Sterilization of the device however proved to be difficult and the set of paper strips had to be replaced repeatedly to provide an optimal inoculum (12)

Wahba's device (12) was more satisfactory but it also employed filter paper strips. Quite unsatisfactory was the arrangement for transfer of indicator strains. Using steel blades may cause an uneven distribution of the indicator strain broth because steel is a non wettable substance. Inoculated with this apparatus the indicator lines are of uneven width (3, 12). The construction has led to the formation of a pocket between some polyvinyl tubes and a centrally located rod which keep the blades in position. This pocket was easily infected and hard to sterilize by the methods employable for the materials in question. Besides the apparatus is unnecessarily complicated to build.

A simple apparatus entirely made of plexi glass holding twelve ridges for the indicator strains will be described.

CONSTRUCTION

The apparatus consists of a stamp and a multiple well tray (Figs 1–3). Exterior measurements of the tray are 7.5 × 4 × 3 cm with one tray at the end 0.9 × 5 × 3 cm. The plexi glass laminae of the outer walls have a thickness of 3 mm, the ridges between the compartments are 2 mm. The stamp laminae are 2 mm, eleven measuring 3.5 × 2.5 cm and one 4.5 × 2.5 cm. The handle is of metal and is screwed into a hole of the 0.3 × 7 × 3.5 (4.5) cm top of the stamp. The pieces may be glued together with contact glue or by chloroform which momentarily dissolves the material.

Since one of the blades is longer than the rest, the stamp fits into the wells in one position only. Thus error in the distribution of indica

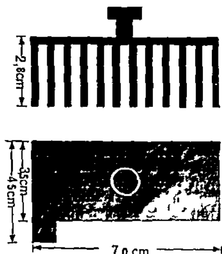


Fig. 1
The stamp. Above: Lateral view. Each plexi glass leaf is painted solidly black. Below: View from above. The white circle outlines the handle.

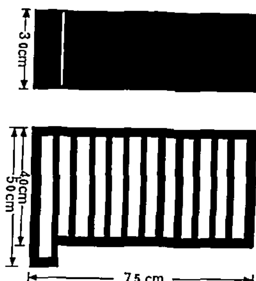


Fig 2

The multiple well tray Above Lateral view The white line indicates the boundary of the longest well Below Plan of the tray seen from above The solid black indicates the plexi glass lamina limiting the twelve compartments

tor strains becomes impossible This also guides in recording results since starting from either end there is a fixed succession of indicators

In order to produce a fully hydrophile surface for even distribution of broth the rough edges of the blades were streaked with pencil lead This only had to be redone after weeks of constant use

Decontamination

The stamp and the tray were rinsed in hot water and Talosan[1] Before use it was exposed to ultraviolet light for three hours This *process was adequate for the purpose The apparatus sustains heat of* 80 C for one hour

Method of Use

The wells were filled with a 5 mm depth of a standard optical density (O D) broth suspension of the indicator strains of logarithmic growth phase Such a volume sufficed for about *200* plates More broth at a time should be avoided since that increases the chances that the inoculum will be excessive and involves a danger of spilling drops of liquid Excess fluid on the stamp will run off when the stamp in an oblique position touches the walls of the wells on its way out

1 A 16.3 per cent parachlormetacresole product with soap and 16.5 per cent concentrated ethanol

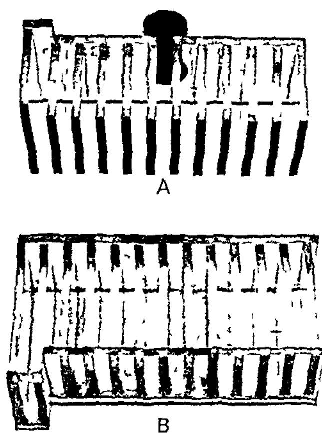


Fig 3
The typing apparatus A The stamp B The multiple well tray

Agar plates (1 5 per cent *agar*) are inoculated with the twelve lines of indicators simultaneously by gently touching the surface Two plates can be attended to before recharging

DISCUSSION

This apparatus allows a rapid transfer of the indicator strains With single loops it is impossible to assure constant distance between the lines and the dose of indicator bacteria is inconstant and uneven along each line Filling the wells with a suspension of given O D one is assured of equal quantities Similarly there is an even distribution of the bacteria along the blades *since they are [illegible] there was no ten*dency towards a dropwise transfer Such [illegible] ns reduce risks of technical error With this apparatus hundred plates could easily be inoculated within an *hour*

SUMMARY

The construction and usage of a new contrivance for bacteriocine typing is presented The apparatus is made entirely out of plexi glass It is simpler to build and allows for more efficient sterilization than other set ups It has been used for pyocine typing of *Pseudomonas aeruginosa*

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TYPING OF *PSEUDOMONAS AERUGINOSA* BY PYOCINE PRODUCTION

By

TOM BERGAN

Received 12 vii 67

In 1925 *Gratia* (8) reported a phenomenon of growth inhibition of one strain of *Escherichia coli* by another The author pointed out the resemblance to bacteriophage lysis The effect was caused by a growth inhibiting substance called colicine (5)

Colicine production is exhibited by all the *Enterobacteriaceae* and has been used successfully in the typing of *Shigella sonnei* (1)

In 1954 *Jacob* (13) published his observations on the synthesis of pyocine an antibiotic like substance by *Pseudomonas aeruginosa* This is a protein or possesses a proteinaceous fraction and is liberated upon the death and lysis of the bacteria (9 13) It is not dialyzeable (9 13) but diffuses through agar gel in the concentrations commonly used for bacteriological media Pyocine is resistant to deoxyribonuclease and may or may not be inactivated by trypsin and pepsin (9 20) It is inactivated by heat at 75 C and is precipitated by ammonium sulphate (9 13) It is resistant to two hours exposure of ultraviolet light as opposed to phage T2 which is inactivated in ten seconds under the same conditions (13) It has in common with phage that liberation of pyocine is increased by ultraviolet light (9 13) Indeed the colicinogenic factors governing colicine production appear as episomes or as autonomous genetic elements (6) Recently the morphology of what appears to be pyocines has been demonstrated by electron microscopy (10)

Colicine and pyocine are closely related substances They have similar physical properties and their action depends upon specific receptors of the bacterial cells The generic term bacteriocines has been accepted for such substances (14)

Pyocine seems to be lethal not bacteriostatic (9) It is active against Pseudomonas whereas *E coli Salmonella* (*typhi* and *paratyphi B*) *Shigella* and *Proteus* are resistant to pyocine in the S but not in the R forms (9) It is noteworthy that colicine has no effect upon Pseudomonas

Holloway (11) studied pyocinogenicity and lysogenicity and suggested that pyocine production might be useful for epidemiological pur

poses *Papavassiliou* in 1961 (18) reported his attempts to establish a pyocine typing system He demonstrated reciprocal growth inhibition of crossing streaks of *Pseudomonas aeruginosa* Pointing out several of the difficulties involved he was not able to propose a formal typing system *Wahba* (20) studied conditions for optimal pyocine production and together with *Darrell* published a technique for pyocine typing (3)

The purpose of this study was to apply pyocine typing to a material of Norwegian hospital strains However differentiation and reproducibility of the system (3) proved to be somewhat crude Attempts were consequently made to analyse and modify the method to render it more sensitive and dependable

MATERIALS AND METHODS

Producer strains were obtained from the diagnostic routine in this institute 87 strains are included in this collection

Identification of strains Pseudomonas were isolated on blood agar using Tryptose Blood Agar Base (*Oxoid*) with 5 per cent human blood added Lactose brom thymol blue plates with 2 5 per cent agar showed lack of lactose utilization The colonies were observed for typical appearance and characteristic smell *Gram* stain were made the oxidase reaction was tested (16) and growth at 42 C was observed Resistance to antibiotics was assayed by a disc method (4)

Biochemical properties were recorded after four days at 37 C and examined as follows

1 Ability to synthesize indole from tryptophane using a casein broth (15)
2 Methyl red and *Voges Proskauer*s reactions according to *Barritt* (15)
3 Use of citrate as sole source of carbon in *Koser*s medium (15)
4 Production of urease on *Christensen*s medium (15)
5 Reduction of nitrate When no reaction occurred after the addition of the reactants zinc powder was added to test the presence of nitrate (15)
6 Production of H S in a meat extract peptone agar medium with 0 1 per cent lead acetate
7 *Liquefaction of gelatin in broth with 15 per cent gelatin*
8 Glucose fermentation was tested in *Hugh* & *Leifson*s medium in tubes (12)
9 Growth and red colonies were observed as described by *Selenka* on a slant nutrient agar with triphenyltetrazoliumchloride (TTC) (19)
10 Ability to produce pyocyanin pyorubrin and pyofluorescein was controlled on *King*s media (15)

Indicator strains were kindly furnished by Dr *M T Parker* Director of the Cross Infection Reference Laboratory Central Public Health Laboratory London They were labelled A52 – B10 – B90 – B39 – M283 – S17 – 8/39 – 10/55 – 148 577 – 594 593 The strains had been selected by *Darrell* & *Wahba* (3 1) seven of these strains *had been mentioned in earlier reports To keep growth characteristics and pyocine* susceptibility as constant as possible the strains were transferred weekly using Tryptose Blood Agar Base (*Oxoid*) slant Eighteen hour slant cultures were used for assays

Standardization of indicator strain inoculum The density of the glucose broth suspension of indicator strains was fixed to produce adjoining but separate colonies This was obtained by suspending a loopful of a 24 hour slant culture into 3 0 ml of glucose broth The bacteria were emulsified with a Pasteur pipette which was emptied completely and rinsed out successively in another two tubes of broth The third tube should read 0 005 0 007 O D on a *Beckman* Model C Colorimeter with green filter of 510–580 mμ To avoid error the third broth tube was used for blank *and left in the same position in the colorimeter while the pipette was rinsed out in it*

Medium for typing is described by *Darrell* & *Wahba* (3)

Pyocine typing technique The strain to be typed was streaked out diametrically

across the plate using a 4 mm loop and incubated at 37 C (3) for 24 hours Chloroform (ca 0 9 ml) was then poured into the lid of the Petri dish and the agar exposed to the vapour for 30 minutes To remove the streak of bacterial growth a 1 cm strip of the agar was removed Subsequently the plate was exposed to $CHCl_3$ vapour for another half hour and finally to air for one hour to evaporate the chloroform Then the plates were inoculated by an apparatus described earlier (?) with the twelve indicator strains and incubated for 18 hours at 37 C before reading

RESULTS

Width of the primary streak Using loops of 0 2 0 4 and 1 0 cm diameter the width of the streak of Pseudomonas to be typed was varied A 0 4 cm width was found to be optimal

Pyocine inactivators A study of the influence of unspecific bacterial substances was undertaken When chloroform killed colonies were painted on agar without leaving visible trace there was growth inhibition of susceptible indicator strains in the area painted Likewise findings obtained by scraping off the bacterial growth were compared with those obtained when the chloroform killed growth was left untouched The zones of inhibition were narrower in the latter case

Also circular agar patches with chloroform killed dense 24 hour growth of hospital strains were placed on the surface of an agar plate some with the bacterial growth turned up and some with it turned down The agar patches were 1 cm high and 1 cm in diameter The plates were inoculated with indicator strain suspensions of such a density that discrete but densely situated colonies would emerge After 3 hours preincubation at room temperature the plates were exposed to 37 C for 18 hours The patches with the killed bacterial growth down were then surrounded by zones of better growth than the patches with the layer of killed bacteria turned up This occurred even when the combinations of pyocin producer/indicator strain had given negative reactions by *Darrell & Wahba*s method (3)

These observations indicated that the killed bacteria contained pyocine inactivating or inhibiting substances It was therefore conceivable that macroscopically unnoticeable remnants of the chloroform killed hospital strains could influence the results of pyocine typing by inactivating the pyocine It has also been shown that Pseudomonas produce other growth inhibiting substances than pyocine so that remnants could even cause stronger inhibition Therefore to avoid unspecific interference with indicator strain growth all traces of the killed hospital strain would have to be removed completely [illegible] ing method developed

Removal of chloroform killed Pseudomonas By [illegible] methods (1 3 7 21) the diametrical streak was scraped off [illegible] glass slide However some material inevitably became [illegible] on the rough surface appearing after scraping off the Pseudomonas since it adhered firmly to the agar used Consequently errors are possibly introduced with the scraping technique Besides scraping is time consuming and

TABLE 1

Results a) of Removing the Chloroform Killed Pseudomonas in Different Ways and b) of Varying Indicator Strain Growth Density

Hospital Strain Typed	Indicator Growth	Treatment of Chloroform Killed Strains: 1 Scraped	2 Not Removed	3 Well Method	4 Filter Paper Strip Growth	5 Lysine Agar Strip Transferred	Comment
7047/65	A	+	—	—	—	—	Inoculum B is better than A With nearly confluent growth treatment 1 showed more inhibition than 3 but a pattern different from that obtained by treatments 3 and 5
	B	++	++	+++	+++	+++	
7317/65	A	++	—	+	—	—	Inoculum B renders the best differentiation Treatments 3 and 5 gave comparable results Technical difficulties with the filter paper prevented assessment on plate 4B
	B	++	++	+++	C	+++	
7441/65	A	—	C	++	++	—	The well method with nearly confluent growth gave better differentiation than with dense growth Plate A2 had inhibition zones different from other plates
	B	++	++	+++	+++	+++	
7493/65	A	—	—	—	—	—	Treatments 3 and 5 gave good differentiation and wide inhibition zones Transition zones were more evident with treatment 1 than with 4
	B	++	++	++	++	++	
8160 65	A	+	+	+	—	—	Plate 3B had better differentiation than 5B The latter had less inhibition
	B	++	+	+++	+++	+++	
8904/6	A	+	+	+	+	+	The most satisfactory plates were with nearly confluent growth and treatments 3 and 5 Plate 1B showed an inhibition pattern different from 3B and 5B
	B	+++	++	+++	++	+++	
8916 6	A	+	-	++	++	—	Plate 3B showed better differentiation and wider inhibition zones than 3A
	B	++	++	+++	+++	+++	

4. A sterile filter paper strip was placed on the agar surface and streaked with Pseudomonas. After 24 hours the strip was simply taken off to remove the bacteria growing on top of it.
5. A plate was heavily seeded with Pseudomonas. After 24 hours a 1 cm strip was cut out and transferred to a second plate inoculated in advance with the indicator strains.

The results are listed in Table 1. To summarize, the well and the agar strip methods yielded the most satisfactory differentiation and wider inhibition zones. Removal of agar to produce a trough is considerably simpler to perform than the transfer of a thin strip of agar to another plate. The former method was therefore adopted as a standard procedure.

Density of indicator strain growth. Previous methods for bacteriocine typing have employed a dense growth of the indicator strains (1, 3, 7, 18). Application of *Darrell & Wahba's* method (3) to Norwegian hospital strains of *Pseudomonas aeruginosa* rendered in my hands poor differentiation, mostly without inhibition or with equal inhibition of all indicators. Some reactions were difficult to interpret as either positive or negative, yielding poorly reproducible results. In assays of *resistance to commercial antibiotics it is accepted that a variation in* inoculum influences the results (4). In a tube dilution technique, MIC appears at a higher concentration of the substance when the number of bacteria per ml is increased considerably. For the disc technique of *Ericsson* (4) the optimum inoculate is nearly confluent growth of colonies.

To investigate the optimal inoculum of indicator strains, duplicates *of the plates listed (1–5) in the preceding section were inoculated with* indicator strains in two concentrations:

(A) gave rise to nearly confluent colonies and
(B) *produced a dense growth ad modum Wahba* (21).

The results are recorded in Table 1. Nearly confluent growth showed inhibition in combinations where a dense growth was not influenced at *all. The former also gave a better differentiation with varying zones of* inhibition from one indicator streak to another on the same plate. Therefore nearly confluent growth was considered optimal and adopted for the standard typing procedure.

Time for indicator strain incubation. An arrangement was set up whereby the pyocine diffused from circular one centimeter diameter patches of agar with chloroform killed 24 hour cultures of hospital strains. These were *placed on plates* inoculated by flooding the indicator strains listed in Table 2. Triplicates of all combinations were put up with the bacterial growth of the patches down as well as up to see whether any difference in zones of inhibition would result. The plates were read at 12, 18, 24 and 36 hours.

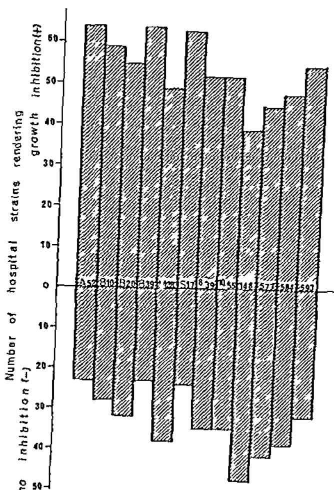


Fig 2

Distribution of positive and negative inhibition of the indicator strains for pyocine typing Each column represents one indicator strain

mm giving a 1 4–1 8 mm diameter of the zones of inhibition when measured according to (4)

Recording The results seem to warrant a simple separation into negative and positive reactions An arbitrary division line at 2 5 cm seemed adequate

Inhibition zones ≧ 2 5 cm are recorded as +
Inhibition zones < 2 5 cm are recorded as —

Using nearly confluent colony growth there is a transition zone of 2 3 mm between no growth and no inhibition Only the complete inhibition is measured

Typing of Pseudomonas hospital strains Typing of 87 strains was carried out according to the technique outlined above All strains have

been typed 2–4 times Type distribution is shown in Table 3 The different susceptibility of the indicator strains to pyocine is evidenced from Table 3 and Fig 2

DISCUSSION

The nearly confluent growth of indicator strains should render this technique more sensitive to pyocine and allow for a greater differentiation than otherwise seen when dense growth is employed Comparisons of results from different days should also become more valid if the inocula were standardized Technical errors are reduced further by removing the strain to be typed *in toto* with the underlying agar This prevents interference from minute amounts of unspecific substances from the killed bacteria

However none of these measures were sufficient to eliminate the variability that could be observed from time to time (Table 4) and which was also seen with the original method (3)

TABLE 4
Variation of Pyocine Typing Pattern by the Same Strains

Hospital Strains	Indicator Strains A 52	B 10	B 20	B 39	M 283	S 17	8/39	10/55	148	577	584	593
1108 /65	0	2 5	2	2	0	0	0	0	0	0	0	0
	3	2 5	2 5	2 5	2	3 5	2 5	2 5	2	2 5	3	3
	3	3	2 5	2	0	0	0	0	0	0	0	0
	3 5	3 5	2 5	2	1 5	3 5	2	2 5	2	2 5	2	3
	+	+	—	—	—	—	—	—	—	—	—	—
9703/65	2	3	2	2	1 5	2 5	1 5	0	0	0	0	1
	2	2	2	2	2	2	2	2	2	2	2	3
	2 5	3 5	2	2	2	2 5	2	1 5	2	2	2	3 5
	2 5	2 5	2	2 5	2	2	2	2	2	1	1	2 5
	—	+	—	—	—	—	—	—	—	—	—	+

The examples show the reactions of two randomly selected samples which were typed four times The numbers indicate zone of inhibition in centimeters approximated to the nearest half centimeter At the bottom is listed the resulting pyocine type of the strain

\+ = Inhibition zone wider than 2 5 cm — = Inhibition zone less than 2 5 cm

Darrell & Wahba (3) were able to separate the strains into 12 groups *Gillies & Govan* (7) used 8 indicator ns and observed 36 subdivisions I found 27 patterns with Wahl r trains Approximately half of the strains inhibit d l mai and ca 1/5 gave negative reactions in all cases

Darrell & Wahba (3) reported a small p c rt of discrepancy *Gillies & Govan* (7) stated that they saw diff rent pyocine types from the same specimens but it is difficult to assess whether that might be

due to methodological inadequacies After 2688 strains had been typed partly by *Darrell & Wahba's* method (3) partly using 32 C incubation and their own eight indicator strains these authors found that further studies are required to confirm its [the method's] reliability They considered the method sufficiently consistent to be a valuable asset The reproducibility of pyocine typing should be evaluated more closely with the indicator strains selected by *Gillies & Govan* (7) to establish whether those might be better suited than the strains employed here

The consistency of pyocine typing is also dependent on the stability of the indicator strains It has been pointed out earlier that their pyocine susceptibility did vary with time requiring periodical typing of the indicators themselves to ascertain that their spectrum is maintained

From Fig 2 it is seen that the indicators had markedly different pyocine susceptibility Strains A52 B39 and S17 were only slightly resistant showing reactions of inhibition with approximately ¾ (73 5 per cent) of the hospital strains It is likely that such strains might be less valuable as tools for a separation into different categories than strains like 148 577 and 584 with their almost 50–50 distribution With this collection of hospital strains however these two sets of indicators have both been equally valuable Within each set of three strains there was different sensitivity patterns to eleven hospital strains (Table 3)

Osman (17) has devised another procedure for pyocine testing but this method is rather laborious for routine use This consists of depositing drops of pyocine on plates much like phage suspensions in phage typing *The main objection is however that the indicator strains* could easily start to liberate phage (due to previous lysogenization or simple contamination) this would alter the reactions but might not be *detectable The morphology of inhibition of growth due to phage or to* pyocine is almost identical

The advantage of pyocine typing is that it entails little maintenance *work and is consequently* simpler than phage and serotyping It may be used under relatively primitive conditions From the findings reported I would however recommend that this method be used merely as a supplement to other methods It is imperative that each strain be *typed more* than once (Table 4) At least this applies to the indicator strains used here

SUMMARY

An attempt was made to elaborate a more sensitive method for pyocine typing It was found necessary to make certain that all the bacterial material was removed before indicator strain inoculation Therefore the strip of agar on which the Pseudomonas grew was removed *in toto* When seeded with a special apparatus the indicator strains rendered better differentiation when using bacterial suspensions of standard density yielding nearly confluent growth

Pyocine typing is recommended for use only in conjunction with other epidemiological typing systems Repeated typing of the same strain is advised

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RAPID IDENTIFICATION OF SMALL NUMBERS OF BACTERIA AFTER SHORT TIME CULTIVATION IN THE PRESENCE OF FITC—CONJUGATED ANTISERUM

Report on a Method

By

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Received 9.viii.67

Small numbers of bacteria present in a liquid can be detected by filtering the liquid through a membrane filter and staining the filter either with conventional stains (1, 2) or with fluorescent antibody (FA) (3). While the former method allows only quantitative estimation of the bacteria present, staining with FA also enables the investigator to identify the bacteria serologically. However, neither method gives him any information about the viability of the bacteria.

Danielsson (4) introduced a two-step enrichment procedure which allows identification of as few as 50–2 living bacteria per sample. He filtered the sample through a membrane filter and incubated the filter in broth at 37° C for a few hours. The broth was then filtered through a non-fluorescent membrane filter on which bacteria could be detected after staining with FA. *Danielsson* states that the method functions well for mixed cultures. This refers, however, to an experiment where *Shigella guanabara* and two strains of *E. coli* were used as test organisms. If instead one intends to identify a mixture of bacteria with greatly differing growth rates, the method would appear to be less well suited. Due to great sensitivity, methods based on enrichment also imply a risk that even minute contamination may interfere with the results.

To avoid the possible disadvantages of enrichment culture, it seemed worthwhile to try to develop an equally sensitive method based on short time cultivation of bacteria on solid media. The use of FA technique would allow identification of small numbers of microcolonies at an early stage of the incubation. FA staining of microcolonies had previously been done either with intact colonies on non-fluorescent membrane filters (5) or with heat-fixed impressions of colonies on glass slides (6).

The present article describes a method based on short time cultivation in the presence of fluorescein isothiocyanate (FITC)—conjugated

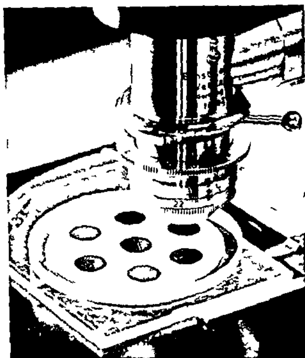


Fig 3
Incubated filters ready for microscopy

see th t the conjugate vas evenly distributed in the FA disks to obtain as nearly as possible the original concentration f conjugate in the disks Originally the entire membrane filter was placed upon the nutrient dish Later I found it more convenient to stamp out small circular pieces of filter about 10 mm in diameter and to put one piece on top of each FA disk One piece was put in the centre where there was no FA to serve as a blank (Fig 1 right) Finally the plastic b x was closed (Fig 2) and incubated at 37 C

L. *Fluorescence Mi roscopy*

A Leitz Ortholux microscope with an Osram HB 200 lamp was used Most often a combination of primary filter UG 1 thickness 1 mm and secondary filter K 430 was used as filter system To be able to inspect the black membrane filters the microscope had to be fitted with equipment for incident illumination (Ultropak) A dry 50 x objective was u ed to look for single bacteria and the smallest micro colonies When searching for the omewhat bigger micro colonies less powerful ob jectives could be used the least powerful a ail ble being a 6 5 x C ndensati n of humidity n the surface len of the object ve as t mj n l r sent but I I not c n titute any serious problem All objectives ver mb nation th a 10 x p ir of oculars

Immediately prior to mi ro co th pla ti l f he in ubator the cover remove l and the lid pla e l a s ree f lly made to f t the stage of the microscope (Fig 3) Because th l wl ile it vas under the m cros ope the reaction with al uld apidly be tested

gently with PBS. The information given in Table 1, however, refe
to untreated filters.

Cultivation in the presence of FA was not found to be inferior
other techniques regarding specificity of the staining reaction. As c
be seen from Table 2, the specificity was entirely satisfactory exce
when the filters were contaminated with staphylococci which fluore
ced weakly with all the sera tested. This fluorescence, however, w
not detectable during the first 2–4 hours of cultivation and caused i
confusion.

TABLE 2

Staining Reaction after 2–4 Hours of Incubation between the Different Test Strai and Several FITC Conjugated Antisera Diluted 1:12

Microbe	Antiserum				
	15	16	14	26	Normal
H. i. type c	+	—	—	—	—
H. i. type d	—	+	—	—	—
Pasteurella haem. KA 1497/60	—	—	—	—	—
Pasteurella mult.	—	—	+	—	—
Proteus rettgeri KB 492 KB	—	—	—	+	—
Proteus rettgeri KB 3200	—	—	—	—	—
Staphylococci	(+)	(+)	(+)	(+)	(+)

Serum 14 was produced by immunization with the present strain of Pasteurell multocida.

DISCUSSION

A method has been elaborated that permits rapid identification o
bacteria after cultivation in the presence of fluorescent antibody. Th
bacteria were trapped on non-fluorescent membrane filters and th
filters incubated on nutrient disks containing both FA conjugate and
liquid growth medium. During incubation the FA conjugate together
with medium components diffused to the filter surface where i
reacted with antigen and became fixed, first to single bacteria, later to
microcolonies. This resulted in progressive concentration of FA and
led to the development of brightly and specifically stained microcolo-
nies that could be detected in the fluorescence microscope after 2–4
hours. As one might expect, FA-stained microcolonies were more
easily discovered on the filter than single bacteria similarly stained.

The sensitivity of the method in terms of the minimum number of
bacteria detectable was not established in the present study, but ob-
viously it is dependent on the volume of the sample to be investigated.
Small sample volumes are naturally advantageous as they permit th
use of small filter diameters which again lead to a better concentration
of bacteria on the filter surface, making a small number easier to de

tect If sample volumes are less than 100 ml filters 10–12 mm in diameter could probably be used permitting numbers in the order of ten bacteria per sample to be detected

The present method permits identification of microcolonies more rapidly than otherwise possible when staining is done *after* cultivation The investigator may also in order to be able to see the microbes as soon as they become detectable inspect the filters repeatedly during cultivation without additional time consuming staining procedures Great efforts have been exerted to make the method simple as well as rapid In this respect the arrangement shown in Figs 1–3 seems to offer a certain advantage However the stability of dried FA conjugate in disks of filter paper remains to be investigated (no definite fall in activity was seen after 6–8 weeks)

Although the method functioned well with the bacteria tested it must be stressed that only a small number of strains all Gram negative has been used If the method proves reliable in a more extensive study it might become a valuable supplement to several techniques in present use not only as a rapid means of identifying bacteria present in small numbers but also as an aid to the early diagnosis of slowly growing pathogenic bacteria It would also seem to offer a simple way to sero type certain bacteria by means of FA technique

SUMMARY

A fluorescent antibody method is described that permits rapid identification of small numbers of bacteria trapped on a non fluorescent membrane filter The microbes were cultured on the filter soaked with growth medium containing FA conjugate In 2–4 hours specifically stained microcolonies developed which could be detected in the fluorescence microscope The sensitivity limitations of the method are briefly discussed and some possible uses are mentioned

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THE NATURE OF RABBIT ANTIBODIES AGAINST HOST COMPONENT OF INFLUENZA VIRUS

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Received 13 vii 67

The haemagglutination (HA) caused by chicken allantoic influenza virus can be inhibited to high titres by antiserum to non infected chicken allantoic fluid The allantoic fluid blocks this haemagglutination inhibition (HI) when added to the antiserum beforehand (2 9) The allantoic antigen responsible for this serological cross reactivity has been purified and was found to be a sulphated mucopolysaccharide (7 8) It is assayed serologically by its ability to block HI antibodies in the haemagglutination inhibition blocking (HIB) test The purified antigen and the host component of the viral haemagglutinin show complete serological cross reactivity Being the only host component of the virus which reacts in the HI test (6 10) the allantoic antigen will be referred to as the host antigen The same host antigen has been demonstrated in high contents in the bile of embryos as well as of adult chickens (4) It has also been demonstrated serologically in chicken amniotic fluid No other tissues have been found to contain this substance (3)

HI and virus neutralizing antibodies produced in mice and rabbits by immunization with influenza virus were studied by *Styk et al* (20) The antibodies were separated by gel filtration on Sephadex G 200 and by ultracentrifugation and they were found mainly in the 7S region In order to detect the 7S antibodies which appear early they added a normal serum cofactor which was not present in the 7S fractions being a high molecular weight substance (16 18) The cofactor was thermolabile and was distinct from known complement factors and normal virus inhibitors (17 19) The mechanism of action of the cofactor was assumed to be an augmented steric hindrance of the antibodies which increased their neutralizing capacity It was also assumed that the cofactor increased the inciden e of collisions between antibody molecules and viral particles thus sh f in the equilibrium of formation and dissociation of ant gen antibod complexes in favour of complex formation (15)

The aim of the present work was t s u t th nature of HI antibodies

to the host component of allantoically grown influenza virus their dependence on cofactor and their relation to precipitating antibodies

MATERIALS AND METHODS

Virus strains The two prototype influenza A and B strains PR 8 and Lee were employed throughout the present study For use in HA and HI tests the virus was purified by adsorption to and elution from chicken erythrocytes in order to eliminate allantoic fluid contamination

The host antigen was purified as described in (7)

Antisera Most experiments were carried out with two rabbit antisera one against allantoic PR 8 virus and one against purified host antigen The antiserum against PR 8 was produced by three subcutaneous inoculations with allantoic PR 8 mixed with Freund s complete adjuvant one ml of each The interval between the two first injections was 14 days the third dose being given two months later The antiserum against purified host antigen was obtained by giving one intracutaneous dose of 0 2 mg of the substance in Freund s complete adjuvant The animals were bled three weeks after the last injection

Freund s adjuvant was prepared with tubercle bacilli grown on an egg free medium (Sauton)

The HI test was performed against 4 agglutination units of the virus and with an 0 5 per cent chicken erythrocyte suspension The antisera were pre treated with crude RDE (Cholera filtrate Philips Duphar Amsterdam) thereafter heated at 56 C for one hour and absorbed in the cold with 10 per cent chicken erythrocyte In order to check for non specific HA inhibitors the serum fractions were first absorbed with host antigen and then examined in the HI test against B/Lee virus The fractions were also checked for haemagglutinins If necessary treatment with RDE and absorption were performed as above HI antibodies to the host component of the virus were titrated against the B/Lee strain since the host antigen is the only substance which cross reacts serologically in this test

The HIB test was used to titrate the host antigen and was carried out as described in (3 5) with 4 HA doses of virus and 4 HI doses of the serum

The diluent in all titrations was saline buffered with phosphate (PBS) 0 01 M to pH 7 2 Cofactor prepared as described below was added to the PBS when this was used for dilution of sera or serum fractions in the HI test

Cofactor The source of the cofactor was normal guinea pig serum In pilot tests the guinea pig serum was found to give maximal antibody enhancement up to a dilution of 1 120 In the present experiments a dilution of 1 60 was used The serum had to be pretreated with RDE in the same way as the antisera but without subsequent heating at 56 C in order not to destroy the cofactor Thereafter the serum was thoroughly absorbed with chicken erythrocytes in the cold to remove normal agglutinins and RDE. So treated the guinea pig serum did not interfere with the virus HA When cofactor had been added all titrations were carried out in the cold, the readings being made after two hours For comparison parallel titrations were set up with heat inactivated (30 min 56 C) cofactor as a diluent

Density gradient centrifugation was performed in a Spinco ultracentrifuge Model L/50 a 10–40 per cent sucrose gradient being employed. One ml of serum diluted 1 2 (or 1 3 after mercaptoethanol treatment) was layered over 4 ml of the gradient. The centrifugation was carried out for 18 hours at 35 000 r p m with a swinging bucket type SW 39 For fractions of approximately 0 5 ml were collected after needle puncture at the bottom of the tube The collected fractions were numbered from the bottom upwards A mononucleosis serum served as a control for the position of 19S globulins (fractions 2 and 3) The position of the albumin (fractions 7 and 8) was located by adding one drop of 1 per cent bromophenol blue to the serum before the separation

Gel filtration The gel filtration was carried out in the cold according to *Flodin & Killander* (1) Columns of G 200 (Pharmacia Uppsala Sweden) of 2 [illegible] × 4 [illegible] were employed Ten ml of the dialysed serum was layered on the Sephadex bed and fractions of approximately 4 ml were collected at a flow rate of about 2 [illegible] per hour

The fractions were examined serologically for HI activity and precipitin [illegible] the optical density at 280 mμ was recorded.

Chromatography on DEAE (diethylaminoethyl) cellulose DEAE cellulose (p a Serva Heidelberg) was employed with columns of 2 6 × 30 cm Elution was achieved through three successive steps 0 01 M phosphate buffer pH 8 0 1 M NaCl in phosphate buffer and 0 3 M NaCl in phosphate buffer Five ml fractions were collected at a flow rate of about 25 ml per hour

The fractions were examined as mentioned under Gel filtration above

The quantitative precipitation test was performed to establish the equivalence zone for the absorptions Portions of 0 25-0 5 ml of serum or serum fractions were mixed with equal amounts of various dilutions of the host antigen in PBS After incubation for 30 min at 37 C and 3 to 6 days at 4 C the tubes were centrifugated at 18 00 × g for 90 min in the cold The precipitates were washed twice with cold saline and thereafter dissolved in alkali for antibody protein analysis by the Lowry modification of the Folin test (11) The antigen protein content was so low that it did not influence the results The supernatants were tested for excess of anti body or antigen in the ring test and in the HI and HIB tests

Double diffusion in agar was performed according to *Ouchterlony* (13 14) Six circular wells were arranged around one central well all with a diameter of 6 mm and 4 to 5 mm apart Since the 19S antibodies diffuse slowly in agar the respective fractions were applied to the wells 1 to 2 hours before addition of the antigen and the other serum fractions Unless otherwise stated the antisera and serum fractions were tested against 0 1 mg per ml of purified host antigen

Mercaptoethanol (ME) treatment ME in PBS (2 Mercaptoethanol Fluka AG Switzerland) was mixed with equal volume of serum to yield a final molarity of 0 25 The mixture was incubated at 37 C for 2 hours The reduction was stopped by adding an equal volume of freshly prepared iodacetamide (Fluka) in 10 per cent molar excess Dialysis was thereafter carried out in the cold overnight

EXPERIMENTS AND RESULTS

The two immune sera were examined for viral HI antibodies against B/Lee virus and by agar precipitation against purified host antigen Both sera gave an HI titre of 1 1024 to 1 2048 They also produced the two precipitation lines described in an earlier publication (7) one near the serum basin sharp thin and strongly concave towards the basin the other midway between the two basins wide and more diffuse and straight

After ME treatment of the sera the HI titres were reduced about four fold The sharp precipitation line near the serum basin could no longer be demonstrated while the other line remained unchanged This show that the macroglobulins are the most potent HI antibodies in these sera and also that they produce the thin sharp line near the serum basin Seven other antisera against allantoic PR 8 were examined in the same way All of these gave lower HI titres after ME treatment the decrease varying from 4 fold to 16 fold

After density gradient centrifugation of serum the fractions were tested for HI antibodies with and without cofa[illegible] The results are re corded in Fig 1 which shows that strong HI antibodies to the host component of the virus were present both [illegible] 19S and 7S regions In one serum (anti I R-8) the HI anti[illegible] found predominantly in the 19S region The other serum [illegible] identical 19S and 7S peaks but the latter is obviously inc[illegible]d by some overlapping 19S anti bodies

Fig 1 also shows that the titres of 19S and 7S HI antibodies were

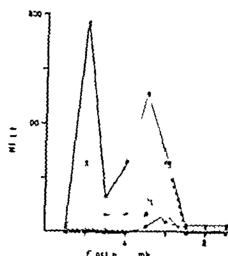


Fig 1

activity of fractions obtained by density gradient ultracentrifugation of an anti... um against the host antigen The effect of ME treatment and addition of cofactor
——— ○ Serum fractions with active cofactor (fresh guinea pig serum)
● Serum fractions with inactivated cofactor (guinea pig serum heated at 56 C for 30 min)
□ ME treated serum fractions with active cofactor
– – ■ ME treated serum fractions with heat inactivated cofactor

re ised when cofactor w is added The incre ise was more marked h 7S antibodies which is in agreement with the findings by *Styk et* (18)

.ltr icentrifug ition of ME tre ited sera showed that the 19S HI ac ty was completely destroyed A final molarity of 0 25 of ME was re red is 0 1 M for 2 hours at 37 C although effective with regard to nonucleosis antibodies left residual 19S HI activities In Fig 1 the es of the 7S fractions may seem somewhat reduced but this may tly be due to destruction of overlapping 19S antibodies as mentioned ve

irus specific antibodies were assayed with PR 8 virus against the i PR 8 serum The serum had first to be absorbed with purified host igen to avoid interference by the strong antibodies to the host iponent The virus specific HI antibodies of this serum were found he 7S fractions exclusively (Fig 2) and showed lower titres than antibodies to the host component Identical results were obtained i nmiotic virus which is free of host antigen (14) and can be used the HI test for virus specific antibodies without absorption of the im with the host antigen

n agar precipit ition the purified host antigen produced the sharp near the serum basin with serum fractions 2 3 and 4 while frac is 5 and 6 gave the more diffuse line midway between the basins s was in accordance with the results of ME-treatment of the whole

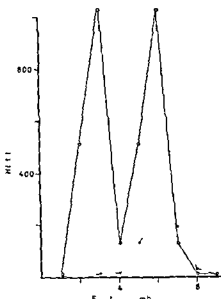


Fig 2

HI activity of density gradient centrifugation fractions of an antiinfluenza A/PR 8 serum The serum was absorbed with the host antigen before testing with PR 8 virus
●--------● HI titres with A/PR 8 virus
O————O HI titres with B/Lee virus

serum and confirmed that the two lines were produced by 19S and 7S antibodies respectively

One of the main purposes of the separation of the antibodies was to ascertain whether the 7S and the 19S precipitins had the same serological specificity

The first question examined was whether the two lines fused in the form of a loop when the respective antibodies were placed in adjacent basins However the different diffusion rates of precipitation of 19S and 7S precipitins and the curved appearance of the line of the 19S antibodies made this difficult It was not possible to obtain lines at the same level and no definite conclusion could be drawn This problem was approached in another manner by absorbing the antigen with separated 7S and 19S antibodies The procedure of the absorption was as described in the quantitative precipitation test with a slight excess of antigen

The absorbed antigen was thereafter tested by agar precipitation against 7S and 19S precipitins respectively and failed to give any precipitation line Controls with the same concentration of unabsorbed antigen gave precipitation lines in agar This indicated that the 19S and 7S antibodies react with the same antigen molecule

DISCUSSION

Allantoically grown influenza B/Lee virus is inhibited in the HI test to high titres by rabbit antisera to allantoic influenza A/PR 8. The cause of this serological cross reaction is the chicken allantoic host antigen described in earlier publications. No other substance has been demonstrated to cross react between these two type strains in the HI test.

The serum against the PR 8 strain of influenza virus used here showed higher HI antibody titres against the host component than against the virus antigen. Usually, however, virus specific antibodies are more potent. When strains isolated from allantois are typed in rabbit immune sera these should first be absorbed with normal allantoic fluid or preferably with the isolated host antigen.

The presence of HI antibodies to the host component of the virus, sometimes even in higher titres than viral specific antibodies, seems to have been overlooked by many investigators. It is uncertain, however, to which extent their results have been influenced by these antibodies because the host antigen present in the virus infected allantoic fluid absorbs at least some of the cross reacting antibody. Normal allantoic fluid from 12 day-old chicken embryos contains about 10 μg per ml of host antigen (8). This amount will permit absorption of 800 HI units if the fluid is used undiluted. The more diluted the fluid the more interference from antibodies to the host component. Purified virus as used here will accordingly be even more sensitive to cross reacting antibodies. Amniotically grown influenza virus has been found to be almost free of the host component (14). This virus is to be preferred in tests for virus specific antibodies if the sera cannot be absorbed with the host antigen.

HI antibodies against the host component of allantoic influenza virus were predominantly of the 19S type in all sera examined, even in sera obtained after prolonged immunization. Virus specific HI antibodies, however, were predominantly or solely of the 7S type, in accordance with findings by other investigators (16).

The 19S antibodies are usually considered to be poor precipitins. In the present double diffusion experiments the 19S precipitins might easily have been overlooked had the serum not been applied some hours prior to the antigen. Since 19S antibodies diffuse slowly, the precipitation may sometimes take place within the serum basins. Another difficulty met with in agar precipitation when both 7S and 19S precipitins are present is that most of the antigen is taken up by the more rapidly migrating 7S antibodies. The 19S precipitation line is readily demonstrated after the antibodies have been separated by ultracentrifugation.

Although the 19S precipitation line was clearly visible, it was rather weak and could only be demonstrated with undiluted sera. When 19S and 7S antibody fractions with the same HI activity were compared for

production of precipitation lines 7S antibodies could be diluted up to 10 times more than 19S antibodies It was to be expected that 19S antibodies would be very active in the HI reaction which is based on mechanical blocking of the virus HA

SUMMARY

1 Antibodies to the host component of influenza virus and the purified antigen were found to be of the 19S and the 7S type in the HI test as well as on agar precipitation

2 The two precipitation lines produced by the purified antigen were shown to be due to 19S and 7S antibodies reacting with the same antigen molecule

3 The virus specific antibodies were found in the 7S region in the serum examined

4 The virus specific HI antibodies as well as the HI antibodies against the host component of the virus were made more potent by the serum cofactor described by *Styk et al* (20)

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TIME SAVING DEVICE FOR READING SENSITIVITY TESTS ON SOLID MEDIA

By

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Received 10 viii 67

Routine methods for determining the susceptibility of bacteria to chemotherapeutics and antibiotics were developed at the end of the 1940s —first the agar cup technique (6 7) and then the disk technique (3 4) The size of the zone of inhibition measured with dividers (6) slide or outside calipers (4) classifies bacterial strains as sensitive fairly sensitive slightly sensitive or resistant usually according to recommendations given by the manufacturers of the antibiotic disks (4) A standard gauge can simplify reading of the sensitivity tests (1) Such a gauge designed for transmitted light has been used routinely in our laboratory since October 1966 The method and its time saving capacity is described below

The agar plate was placed on an engraved perspex gauge illuminated from below It was possible for the examiner immediately to decide whether the strain was sensitive fairly sensitive etc to the antibiotics in question The results were noted in clinical terms

DESCRIPTION OF THE DEVICE

The gauge consists of a piece of perspex 13 × 11 × 0 4 cm with number of *engraved circles (Fig 1) ει* one 9 cm in diamete rre p n l ng t the circumference of the petri dish and two concentric circl for e ch f t e d sk on the plate Slightly sen itive" and resistant st ain a e gener ll s f ed a re si tant" in our outine tests To facilitate read ng or b r le l n cs the diameters of the n ntric cir les are 1 mm horter than those f th es of inhibition corresp nding to the grades sens tive and fai ly s ns t ve re pe tively Thus the zone of inh b it on hould for a susceptible stra n extend at least 0 5 mm outside the engra ed c cle

Two tubul lam in the upper part of a box illuminate the gauge (Fig 2) nd the culture pl t The l ht opening can receive two gauges side by side The e rest on a fillet fa t n d to ne side of the opening and otherwi e only n the edge of it With the lam lo t the top of the box the light impinge upon the gauge at a large angle f in idence The floor of the box is lined with black cloth. A titled position of th box fac litat s work.

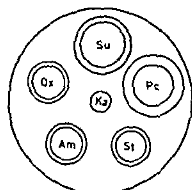


Fig 1
A gauge with zones corresponding to the grades "sensitive" and "fairly sensitive" respectively for sulpha penicillin streptomycin ampicillin oxitetracyclin and the grade "sensitive" for kanamycin

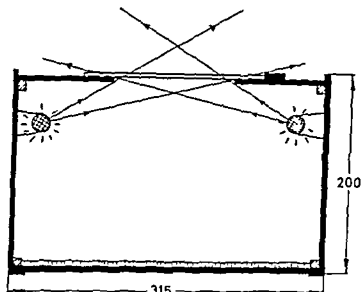


Fig 2
The box used for the illumination of the perspex gauge

RESULTS

The device was used mainly in our urinalysis section In 1966 as many as 30 000 urine samples were received for culture For practical reasons reading of the sensitivity tests by conventional methods with calipers or dividers could no longer be used The measuring of the inhibition zones was very time consuming Even the translation of the diameters of the inhibition zones into clinical terms required at least one hour a day Replacement of the compasses by the gauge meant a re

duction by 50 per cent of the time necessary for reading and recording the results

The results obtained with the gauge sometimes appeared uncertain and were therefore checked by measurement with calipers The frequency of the results judged as uncertain when read with the gauge was studied

In a series of 277 sensitivity tests the results were read with the gauge by one laboratory assistant with calipers by another All strains classified as sensitive or fairly sensitive by one method were classified as such also by the other method In 92 per cent of the cases the gauge allowed a clear cut estimation of the susceptibility In the remaining 8 per cent the judgement was uncertain

DISCUSSION

A report in 1964 (3) based on inquiries at 16 laboratories in different countries recommended measurement of the zones of inhibition with calipers or dividers No information was given about the use of a gauge

In 1965 *Chabbert* gave a report on a gauge printed on white paper with the concentric circles in different colours (1) Using that model no special illumination is required for the reading of the sensitivity tests of strains with dense growth or with pigmented colonies *e g* staphylococci With the use of transmitted light as described in this paper the inhibition zones are well outlined even when growth is scanty

Regarding the illumination of the gauge various designs are possible Thus a model used in immunology (2) can be used for the reading of microbic sensitivity tests with the transparent gauge The size of the light opening however should be adjusted Each microbe is tested with a selected series of chemotherapeutics and antibiotics Such a series is covered by two readily exchangeable gauges side by side on the viewing box

SUMMARY

Routine microbic sensitivity tests in solid transparent media can be read in transmitted light against an engraved perspex gauge In our hands the method required about half the time required if other instruments were used such as proportional compasses outside calipers or slide calipers

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present investigation deals with the problem whether and to what extent deconjugation and 7α dehydroxylation would occur when bacteria capable of such reactions *in vitro* had been established in the intestinal tract of germfree animals

MATERIALS AND METHODS

Animals

Contamination of germfree mice In a preliminary screening trial to contaminate germfree animals 3 male mice 2?0 days old from the germfree NMRI strain on diet D 7 (5) were transferred from the breeding unit in single animal isolators used by *Gustafsson et al* (10) The bacteria were introduced by spraying approximately 1 ml of a 72 hour culture in Todd Hewitt broth (Oxoid) (TH broth) into the isolator by means of a sterile syringe which pierced the air filter At the end of the experiments *i e* after 14 days, the mice were killed by exsanguination under ether anaesthesia The last faecal pellet in the rectum was collected and examined bacteriologically The presence of Strain H was also controlled biochemically using *in vitro* methods (11)

Contamination of germfree rats The rats used were of the Swedish germfree strain and reared according to the technique described by *Gustafsson* (4) They were given semisynthetic diet D 7 and water *ad libitum* (5) Cholic acid 24 C^{14} was administered *per os* as sodium salt dissolved in saline The rats were kept in metabolism cages and faeces were collected and frozen every 24 hours The faeces collected for 4 days were pooled combined and analysed for the labelled compounds excreted.

The bacterial strains were grown in TH broth or Fluid Thioglycollate medium (Difco) (FT medium) Five ml of the cultures were transferred into sterile glass ampules and put into the isolator using techniques described elsewhere (4) The rats were contaminated by giving each animal 1-2 ml of the broth cultures both *per os* and *per rectum* The remaining broth was swabbed on to the skin of the animals as well as being distributed in the diet

Bacteriological Procedures

The strains used for experiments are listed in Table 1 together with their sources and the transformation *in vitro* of bile acids. Quantitative determinations of deconjugating and 7α dehydroxylating microorganisms were performed on faecal samples taken at intervals as described earlier (15)

Chemical Procedures

Extraction methods Faeces were homogenized in 40 ml water Ethanol was added to give a final concentration of 80 per cent and the mixture boiled for 12 hours and filtered The filtrate was evaporated dissolved in water acidified to pH 1 with hydrochloric acid and extracted first with ethyl ether and then with butanol Unconjugated bile acids and glycine conjugates of dihydroxycholanoic acids can be extracted with ether from an acidified aqueous solution whereas glycine conjugates of trihydroxycholanoic acids and all taurine conjugates must be extracted with butanol Therefore the labelled material in faeces was separated into two groups according to extractibility with ether The labelled compounds in these groups were further separated with chromatography

Chromatographic technique For the description of the chromatographic technique and the phase systems used for column chromatography and thin layer chromatography (TLC) see (14)

Fractionation of labelled compounds in ether extracts Preliminary fractionation of labelled metabolites was performed by reversed phase partition chromatography on columns with phase system CI When 200 ml of moving phase had passed the columns the glycine conjugates trihydrox cholanoic acids and their keto derivatives were eluted Dihydroxycholanoic acids and their keto derivatives were retained in the stationary phase and were rechromatographed using phase system FI The isolated labelled fractions were further analysed with TLC to establish the identity of the labelled compounds

Fractionation of labelled compounds in butanol extracts The labelled taurine con

TABLE 1

Names and Sources of Bacteria Used and Their Transformation of Bile Acids in vitro

Bacterial strains	Source†	Transformation of bile acids§				
		Splitting of conjugated bile acids	Removal of 7α hydroxyl group	Oxidation of hydroxyl group at		
				C-3	C-7	C-12
Strain II	DGR	—	+	+	+	+
Bacillus cer us 14/11 63	SBL	—	—	+	+	—
Bacillus subtilis ATCC 6633	ATCC	—	—	—	—	—
Clostridium G62	DGR	+	—	—	—	—
Clostridium perfringens type B	RVC	+	—	+	—	—
Clostridium perfringens CN 1491	SBI	+	—	+	—	—
Escherichia coli 3201	WW	—	—	—	+	—
Lactobacillus casei ATCC 7469	ATCC	—	—	—	—	—
Lactobacillus delbrueckii ATCC 959	ATCC	—	—	—	—	—
Proteus mirabilis 4198	WW	+	—	—	—	—
Str pt oc us faecal M 19	SBI	+	—	—	—	—
Streptoc cus f ie alis ATCC 8043	ATCC	+	—	—	—	—

The nam f th tr t these given by the reference laboratories
† Abbreviations *ATCC* … Type Culture Collection Washington DC USA *DGR* Department of Germfree Research Stockholm Sweden *RVC* … College Stockholm Sweden *SBL* State Bacteriological Laboratory Solna Sweden *WW* Kar l in W Wilhe m n Bakteriologiske Institutt Oslo Norway
§ The transf rm t f … ent bile acids is published elsewhere (13)
See (8)

jugates were separated as one group from the glycine conjugates by chromatography on columns with phase system C1 The taurine conjugates were hydrolysed in N NaOH for 6 hours at 120 C in a sealed tube After acidification the free bile acids were extracted with ether and analysed with column chromatography and TLC as described above

RESULTS

Establishment of Strain II

Mice The results of the screening trial with contaminations of germ free mice are listed in Table 2 They indicated that it was not possible to establish the fastidious obligately anaerobic 7α-dehydroxylating Strain II as a monocontaminant Strain II might be established however when introduced together with a second contaminant

TABLE 2
Establishment of Strain II in Germfree Mice

Bacterial strains	Strain II in faeces after 14 days
Strain II	—
Strain II + *Bacillus cereus* 14/11 63	+
Strain II + *Bacillus subtilis* ATCC 6633	+
Strain II + *Clostridium perfringens* CN 1491	+
Strain II + *Escherichia coli* 3901	+
Strain II + *Lactobacillus leibrückii* ATCC 9595	+
Strain II + *Proteus mirabilis* 4198	+
Strain II + *Streptococcus faecalis* M 19	—
Strain II + *Streptococcus faecalis* ATCC 8043	—

Rats As shown in Fig 1 establishment of Strain II in germfree rats was not always obtained In trials 1 2 and 3 cultures of faeces were all negative 3 days after contamination except in animal number 1 when a 3 day old culture in TH broth was used However even in this rat all control cultures turned out to be negative after 14 days In trials 4–7 an immediate establishment was obtained in all the animals after introduction of a 3 day old culture in TH broth and Strain II could be recovered for several months as a monocontaminant in these animals

Strain II was easily established in germfree rats when it was introduced with *Streptococcus faecalis* ATCC 8043 or with *S faecalis*, ATCC 8043 and other bacterial strains (Fig 1 trials 8 and 9)

In another series of contamination experiments 2 rats placed in metabolism cages on one side of a large 6 × 6 foot jacket isolator (Fig 2) were infected with faeces from a rat harbouring Strain II as a mono contaminant On the other side of the isolator 2 germfree rats were infected with a 7 day culture of Strain II in TH broth One uninfected animal served as a control on each side of the isolator Strain II became established in both the animals infected with faeces and in the control rat on the same side of the isolator However all the animals in

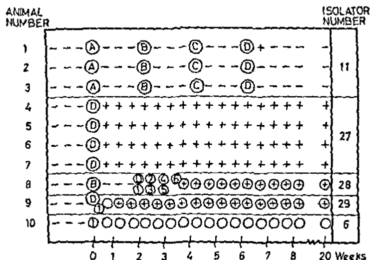


Fig 1

Contamination of germfree rats with different cultures of Strain II either alone or together with other bacteria

Animals no 1-10 were voluntarily contaminated with
(A) = 7 day culture of Strain II in FT medium
(B) = 3 day culture of Strain II in FT medium
(C) = 7 day culture of Strain II in TH broth
(D) = 3 day culture of Strain II in TH broth
(1) = *Streptococcus faecalis* ATCC 8043
(2) = *Lactobacillus casei* ATCC 7469
(3) = *Bacillus cereus* 14/11 63
(4) = *Clostridium perfringens* CN 1491
(5) = *Clostridium* G9?
(6) = *Escherichia coli* 3?01

Examination of faeces was made twice a week (—) no growth (+) growth of Strain II ○ growth of microorganisms 1-6

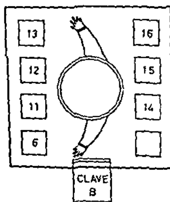


Fig 2

Plan of jacket isolator with metabolism cages
Numbers refer to animals in Fig 3

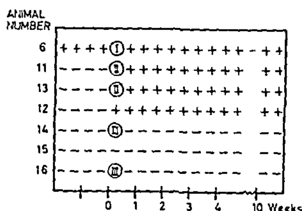


Fig 3

Contamination of germfree rats with Strain I in jacket isolator (Fig 2)
(I) = Rat no 6 (Fig 1) with Strain II established before transfer to isolator
Animals no 11 12 14 and 16 were voluntarily contaminated with
(II) = Faeces from rat no 6
III) = 7 day culture of Strain II in TH broth
Examination of faeces was made twice a week
(—) = no growth (+) = growth of Strain II

ected with the TH broth culture of Strain II and the control rat on the pposite side of the isolator remained germfree for 5 months although hese animals were handled by the same jacket attired attendant as hose in which Strain II had been established with the faecal culture Fig 2 and 3)

Quantitative Determination of 7α dehydroxylating and Deconjugating Microorganisms

The number of the 7α dehydroxylating microorganism (Strain II) resent in the faeces of all mono di and polycontaminated rats was f the same order of magnitude (Table 3) Strain II and the deconjugat ig microorganisms reached approximately the same levels (10^9–10^1) n the faeces of both dicontaminated and polycontaminated rats

Transformation of cholic acid in vivo Cholic acid 24 C^{14} was admini ered orally to conventional germfree mono di and polycontamin ted germfree rats and the nature and amounts of labelled metabolites xcreted in faeces were determined The results are summarized in able 4

Splitting of conjugates The orally administered cholic acid is ab orbed and appears in bile in a conjugated form mainly as taurocholic cid In germfree animals the bile acids are excreted in faeces as con igates whereas in conventional rats the faecal bile acids are mainly nconjugated due to splitting by intestinal microorganisms (6) No plitting was observed in the rat monocontaminated with the 7α ehydroxylating bacterium (Strain II) Splitting occurred in the

germfree rats contaminated with bacteria known to split conjugates (*S faecalis Clostridium perfringens* and *Clostridium*, G62) The extent of hydrolysis (15–25 per cent) however was much smaller than that observed in conventional rats on the same diet

TABLE 3
Establishment of Strain II in Germfree Rats

Contaminating bacterial strains	Animal no	No of bacteria per gm of faeces Deconjugating	7α dehydroxylati
Strain II	7	———	10^{10} 10^{10}
Strain II + *Streptococcus faecalis* ATCC 8043	9	10^{9} 10^{10}	10^{9} 10^{10}
Strain II + *Bacillus cereus* 14/11 G3 + *Clostridium* G62 + *Clostridium perfringens* CN 1491 + *Escherichia coli* 3701 + *Lactobacillus casei* ATCC 7469 + *Streptococcus faecalis* ATCC 8043	8	10^{9} 10^{10}	10^{10} 10^{10}

Two separate readings 5 months after contamination

7α dehydroxylation In the germfree rat with the 7α dehydroxylating bacterium as a monocontaminant no 7α dehydroxylation was observed In the polycontaminated rats Strain II was able to 7α dehydroxylate the formed unconjugated bile acids No deoxycholic acid was found among the conjugated bile acids in any of these rats

Oxidation of hydroxyl groups In the rat monocontaminated with the 7α dehydroxylating bacterium analysis of the conjugates showed the presence of two labelled metabolites of cholic acid The major metabolite had the TLC mobility of 3 12 dihydroxy 7 keto 5β cholanoic acid

DISCUSSION

It has previously been shown that establishment of strictly anaerobic microorganisms such as *Bacteroides* or anaerobic lactobacilli often failed in germfree animals (3 16) Our difficulties concerning the establishment of cultures of Strain II are in agreement with these observations

Strain II was never established when 7 day cultures in TH broth were introduced Our findings indicate that cultivation of Strain II in TH broth for 3 days gives the best result if an establishment as a monocontaminant in the animals is achieved although this method failed in 3 out of the 7 rats investigated Strain II was however easily established when accompanied by other bacteria The fact that Strain II is not able to 7α dehydroxylate conjugated bile acid (14) does not seem to be of any importance for its establishment as it could easily be established in germfree mice with *Escherichia coli Bacillus cereus*

... h a 7α dehydroxylating Bacterium (Strain II) was Established as a Mono Di or ... er Administration of Cholic Acid 24 C[14] was Analysed

	Per cent of labelled compounds in different fractions after chromatography of						
excreted unconjugated bile acids				excreted conjugated bile acids after hydrolysis *in vitro*			
...olic acid	Metabolites of cholic acid with hydroxyl or keto group at C-7	Deoxycholic acid	Metabolites of deoxycholic acid	Unchanged cholic acid	Metabolites of cholic acid with hydroxyl or keto group at C-7	Deoxycholic acid	Metabolites of deoxycholic acid
2	6	72	20	–	–	–	–
–	–	–	–	100	0	0	0
–	–	–	–	87	13	0	0
00	0	0	0	100	0	0	0
55	18	27	0	82	18	0	0
44	16	40	0	83	17	0	0

In germfree rats, bile acids are excreted in faeces in conjugated forms mainly as taurine conjugates (1 6) Strain II is unable to split conjugated bile acids and to form taurodeoxycholate *in vitro*, and the present investigation demonstrates the same inability *in vivo* An additional contamination with deconjugating microorganisms shows that Strain II is able to 7α dehydroxylate the free bile acids formed by this second contaminant However transformation of the labelled cholic acid administered to different metabolites was considerably less than in conventional rats on the same diet (9) This could not be due to the fact that the bacteria had not been established in sufficient large numbers On the contrary the numbers of Strain II in the faeces from the ex germfree rats in this investigation were found to be higher (10[9] versus 10 -10 per gm of faeces) than in the conventional rats on the same diet D7 (15)

Di and polycontaminated rats have not been conventionalized with regard to the quantitative extent of deconjugation and 7α dehydroxylation of bile acids This has not been obtained even though the 7α dehydroxylating bacterium in the intestinal tract of these rats has

attained equal or higher numbers as compared with those in the conventional rat on the same autoclaved diet. Other factors such as intestinal pH, redox potential, symbiosis with other intestinal microorganisms etc. may play important roles for the extent of the intestinal microbial transformation of bile acids.

SUMMARY

A bacterium (tentatively identified as a member of the tribe *Lactobacilleae*) with the *in vitro* ability to 7α dehydroxylate bile acids was established as a monocontaminant in germfree rats. Examination of the faeces of these rats after administration of cholic acid —24-C^{14} showed that bile acid conjugates had not been split or 7α dehydroxylated but had been oxidized to compounds with keto groups at C-7. When deconjugating bacteria had been established in the digestive tract of germfree rats 15–25 per cent of the excreted bile acids were unconjugated. In rats polycontaminated with both deconjugating and 7α dehydroxylating bacteria 7α dehydroxylation of the formed unconjugated bile acids was observed.

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BRIEF REPORT

CADMIUM INDUCED CHANGES IN THE INTRATESTICULAR PRESSURE IN THE RAT

By *Martti Kormano & Outi Suvanto*

The injurious effect of cadmium salts on the mammalian testis and caput epididymidis has been well documented during the past ten years. More recent observations (*Gunn et al* 1963 *Chiquoine* 1964 *Mason et al* 1964 *Macková et al* 1965 *Niemi & Kormano* 1965 *Waites & Setchell* 1966) have made it possible to localize the primary site of action of cadmium salts to the testicular capillary endothelium intertubular oedema and spermatogenic damage being later consequences of the vascular damage An increase in the intratesticular pressure has been reported but not registered after cadmium administration and this increased pressure has been claimed to interfere with blood supply and drainage to produce an ischaemic necrosis of the testis (*Mason et al* 1964) In the present study the intratesticular pressure in the rat shortly after cadmium injection was studied with a view to evaluating the role of pressure changes in the process of cadmium induced testicular necrosis

The U tube manometer used for measurement of intratesticular pressure is described in detail by Sundell (*Sundell 1964*) The fluid was brought into contact with the subalbugineal space through a 17 gauge needle In order to obtain the zero pressure level the apparatus was adjusted to the level of testes to be measured The needle filled with the test fluid was inserted carefully into the testis to avoid damage to the blood vessels A thin metal wire was passed through the needle to remove any obstructions in the tip of the needle After the saline in the manometer was brought to the atmospheric pressure with the syringe communicating with the air space the pressure within the system was slowly raised until the fluid in the adapter and the needle started to move to the tissue This pressure measured in mm of water was considered to represent intratesticular pressure As little as possible of the test fluid was allowed to flow into the tissue

Altogether 10 cadmium injected (0 04 mM/kg) adult Sprague Dawley rats and 6 uninjected control animals were subjected to measurements under light ether anaesthesia 0 2 4 8 24 and 48 hours after the beginning of the experiment The pressures in both testes from each animal were measured

The results are graphically presented in Fig 1 The mean initial pressure was the same in the experimental and the control testes The pressure in the control testes remained approximately constant in every measurement except for a slight lowering at four hours possibly caused by the frequent piercing of the tunica albuginea

The mean intratesticular pressure in cadmium injected animals did not differ significantly from that in the controls at 2 hours but 4 8 24 and 48 hours after cadmium injection the mean intratesticular pressure was significantly ($p < 0.001$) higher than the respective control value

The normal subalbugineal pressure in the rat observed in the present study is of the same order as the intratesticular pressure in the rabbit (*Holstein & Weiss 1967*) In the rabbit rhythmical changes in the intratesticular pressure produced by smooth muscle cells in the tunica albuginea have been observed (*Holstein & Weiss* 1967) Since the tunica albuginea of the rat testis does not contain smooth muscle cells rapid inherent variations in the intratesticular pressure cannot be expected A significant increase in the intratesticular pressure appeared already 4 hours after subcutaneous cadmium injection and the pressure remained high for the whole two day observation period The pressure change seems to set in immediately after the first changes in the capillary wall and hence intensifies the rapidity and the extent of the tissue damage by secondarily interfering with the capillary and venous circulation However there is evidence that the damage to capillary walls is severe

Received 24 1 68 from the *Department of Anatomy University of Helsinki Siltavuorenpenger Helsinki 17 Finland*

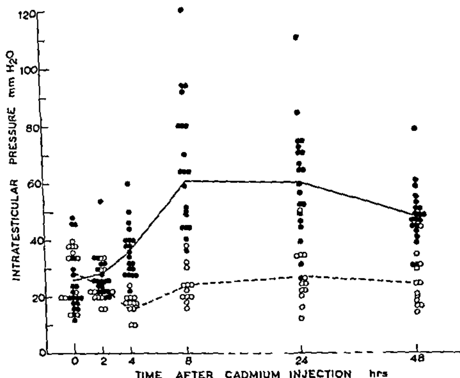


Fig 1

Registration of the intratesticular pres ures in testes from experimental (●) and control (○) rats after subcutaneous cadmium injection

enough to produce nutritional disturbance in the permatogenic epithelium even if the pressure is relieved by opening the tunica albuginea at the time of cadmium injection (*Kormano* & *Niemi* 1965)

Summary

The intrate ticular pressure in the rat was measured at intervals from 0 to 48 hours after a subcutaneous injection of 0 04 mM/kg cadmium chloride 4 hours after the injection the pressure ins de tunica albuginea was found to be significantly elevated. The mean pressure at 8 hours was more than twice the control value The pressure remained significantly elevated for the rest of the two day observation period However direct vascular damage and not pressure changes are sugge ted to be re ponsible of the extensive testicular injury following cadmium chloride in jection

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BRIEF REPORT

TRANSFER OF EXPERIMENTAL MOUSE AMYLOIDOSIS WITH WHOLE SPLEEN GRAFTS BETWEEN SYNGENEIC ANIMALS

By *Finn Hardt*

The transfer of amyloid disease from amyloidotic animals to syngeneic recipients with spleen cell suspensions or homogenates hereof has previously been described (Werdelin & Ranløv 1966 Ranløv & Werdelin 1967 Ranløv 1967) In the present paper transfer of amyloidosis is carried out by means of transplantation of intact spleen tissue from amyloidotic mice to syngeneic non amyloidotic recipients

Methods

The randomized animals derived from our inbred colony of the C3H strain all mice being between 8–10 weeks of age weighing from 23 to 27 gm

Donors 10 mice were given a daily injection of ½ ml of a 5 per cent solution of sodium caseinate subcutaneously for 21 days In addition 5 untreated control donors were included

Transplantation Under anaesthesia (Avertin®) two small slices of spleen were removed from each donor and grafted to right kidneys of two recipients according to the method described by *Wheeler et al* (1966) The remaining part of the donor spleens was kept for histological examination

Recipients The recipients recovered rapidly from the operation (no significant mortality occurred) The grafted animals were kept for 10 days on oats and water On days 11 and 13 each animal was given 0 05 mg of nitrogen mustard (Erasol®) in ½ ml saline subcutaneously in order to accelerate the presumed amyloid formation (*Teilum* 1954) The day after the last nitrogen mustard injection that is on day 14 after the transplantation all recipients were killed

The graft bearing kidney the contralateral kidney liver lung and spleen were fixed in neutral formalin Sections were stained with haematoxylin eosin methyl green pyronine alkaline Congo Red and the P A S stain Amyloid was identified by its morphology and by its birefringence with Congo Red under crossed polars

The degrees of amyloidosis—if any—were evaluated on sections of spleens and spleen grafts according to the semiquantitative method—ranging from 0–6—described by *Christensen & Hjort* (1959)

Results

The main results are outlined in Table 1 From the histological examinations of the donor spleens at the time of grafting it appeared that amyloid was present in all of the casein treated animals in degrees ranging from 2–4 and that no amyloid was found in any of the spleens from the untreated control donors

In the control group amyloidosis developed in one out of 10 animals in the remaining 9 animals no amyloid was found In contrast all of the mice of the experimental group showed a severe and widespread amyloidosis in particular localized to the graft bearing kidney and the host spleen

Discussion

Transfer of amyloidosis has earlier been accomplished by means of spleen cell suspensions and spleen cell homogenates while the present experiment demonstrates a similar transfer with grafts derived from casein treated amyloidotic whole spleens These results feature two pertinent events Firstly reabsorption of the grafted amyloid in the normal recipients did not occur and secondly The amyloidotic

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Received 30 1 68 from *the Rheumatic Research Laboratory the University Institute of Pathological Anatomy (Head G Teilum M D) Copenhagen Denmark*

TABLE 1

	Number of recipients	Type of graft	Mean degree of amyloid in graft (range)	Mean degree of amyloid in host spleen (range)
Experimental group	20	Amyloidotic spleen	2½ (1–4)	3⅓ (3–4)
Controls	10	Normal (non amyloidotic) spleen	0 (0–3)	0 (0–0)

grafts proved effective in inducing generalized amyloidosis in the host tissues thus supporting the concept of a trigger substance or inducer responsible for the initiation of amyloid formation from reticuloendothelial cells (*Ranløv* 1967)

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Östberg Görel TEMPORAL ARTERITIS IN AUTOPSY AND BIOPSY MATERIAL

Hassler O BRAINSTEM HAEMORRHAGES SECONDARY TO EXPANDING SUPRATENTORIAL CONDITIONS

Several recent studies of secondary haemorrhages of the brainstem some of which comprise serial sectioning have indicated the haemorrhages to be of arterial origin (focal arterial necrosis and rupture dissecting haematomas along the arteries) The pathogenesis of such haemorrhages was studied with arterial and venous microangiography The paramedian arteries and veins in the superior part of the brainstem were found to have a divergent more cranio caudal direction than the middle and lower paramedian vessels. They are therefore highly elongated by the caudal displacement of the brainstem that occurs in the presence of an expanding supratentorial condition This stretching is added to the one resulting from the broadening and shortening of the brainstem that also generally occurs in these conditions The two kinds of stretching in combination cause the ruptures In rare cases of expanding supratentorial condition the brain stem is not displaced caudally it is neither broadened nor shortened but flattened against the clivus If so there is no stretching of any paramedian vessels and apparently no haemorrhages

Obel Anna Lisa MORPHOLOGICAL CHANGES IN EXUDATIVE EPIDERMITIS OF PIGS

Pontén J & Thyresson Nils GRANULOMATOUS CHEILITIS—AN EPITHELIOID CELL LYMPHANGITIS

Willén R Berg Rebekka Hansson O Norlbring F Sourander P & Stenram U FATAL VARICELLA GENERALISATA IN A CHILD WITH IMMUNOPATHY AND NEUROLOGICAL RETARDATION

A 4 year old girl with immunopathy (Acta paeditr scand 55 264–272 1966) succumbed in varicella generalisata At autopsy scattered crusted black brown lesions of healing varicella on the skin were observed The lungs were sites of cockade shaped greyish white lesions with a haemorrhagic umbilicated centre and a peripheral haemorrhagic zone In liver pancreas and spleen there were haemorrhagic necroses Light microscopy showed intranuclear inclusion bodies in liver lungs and spleen and by electron microscopy viral particles were demonstrable in these organs as well as in the pancreas The viral particles were of two main types with a hollow centre and with an electron dense centre They were mainly found in the nuclei but were also seen in the cytoplasm close to the nuclear membrane In the cerebrum there was a retarded evolution of nerve cells in the cortex Unspecific

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degeneration was seen in the cerebellum lumbar nerve cells and spinal ganglions Virus isolation and culture on human embryonal cells from heart blood skin and lungs was positive with characteristics of varicella

Henschen F THE FIRST MEETING OF THE SWEDISH PATHOLOGICAL SOCIETY A 50 YEAR ANNIVERSARY

Bergstrand A THE STRUCTURE AND FUNCTION OF THE MICROSOMAL MEMBRANES

Jakobsson Sten STABILITY OF LIVER AND KIDNEY MICROSOMES

Subfractionation of subcellular particles requires an effective homogenization procedure. In this procedure it is of great advantage to obtain spherical particles in order to predict their sedimentation properties The particles also must be constant in their physical chemical and enzymatic properties

Rough and smooth vesicle subfractions of liver microsomes show differences in stability Dilution of the total microsomal fraction gives rise to a severe aggregation of the smooth particles but the rough fraction is stable Aggregation of smooth membranes can be prevented by addition of albumin Rough microsomes are less sensitive to mechanical treatment and show a higher enzyme stability than the smooth counterpart

Renal cortex microsomes have a heterogenous composition containing components both of endoplasmic reticulum and plasma membrane Following homogenization in 0 25 M sucrose the microsomal fraction consists of spherical and elongated particles However when homogenization is performed in diluted buffer at a neutral pH all particles appear in spherical form. Following dilution these spherical microsomes easily aggregate Resuspension of the centrifuged pellet results in total aggregation By centrifugation over a cushion of dense sucrose concentration of the material is achieved without aggregation of vesicles

During differential centrifugation or centrifugation in a stabilizing gradient the size is the greatest factor deciding the sedimentation properties of the particles Renal microsomes are probably fully permeable to sucrose For this reason the density and the hydration water in a sucrose gradient will be of importance but not the particle size By addition of a substance of high molecular weight such as Ficoll which cannot penetrate the particles vesicles with different size can be separated Therefore it is possible to obtain microsomal subfractions from renal cortex which display different properties

Glaumann H FUNCTIONAL RELATIONSHIP AMONG VARIOUS MICROSOMAL MEMBRANES IN RAT LIVER

Liver micro omes separated into rough sm th I an I m th II membranes differ in ultrastru ture and chem cal comp os tion A far a I p ds ar conc rned, it has been po tulat d that the various membranes h n dent l phospholip d composition but that there are differences in neutral lip d comp ition Likewise the membranes differ as to the rate of incorporation of l pid pr ursors The ques ti n theref e arises wl ether the three subfractions derive from the endoplasmic reticulum o wl ether membranes from other cell organelles contaminate the fractions.

Albumin synthesis and transpo t are connected with the endoplasmic reti

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culum and participation in the process indicates relationship with this organelle The albumin transport was studied 5 to 35 minutes after i v injection of ^{14}C-leucine After subfractionation and sonication of the liver microsomal fraction the albumin was isolated immuno electrophoretically At 5 min, most of the radio activity (~ 60 per cent) was recovered in the rough fraction which exhibited almost 6 times more specific activity than the smooth counterparts. The specific activity then increased rapidly and reached its maximum at about 15 min followed by a rapid decrease

The two smooth fractions which displayed an almost identical pattern exhibited a considerably lower activity in the beginning and reached their maximum somewhat later at ~ 20 min The early incorporation of and rapid decrease in activity of the rough fraction along with the initially low but later considerable specific activity of the two smooth fractions support earlier data suggesting a transport of newly synthesized protein from the rough to the smooth part of the endoplasmic reticulum The similar pattern of incorporation of the two smooth fractions indicates that both of these might be part of the same functional system In order to exclude a possible contamination by other cell organelles an enzyme analysis was performed for various "marker enzymes" including lysosomes (acid Pase) plasma membranes (AMPase and K stimulated p nitrophenyl Pase) and mitochondria (mono amino oxidase and cytochrome c oxidase) Only minimal parts of the total activities were recovered in the three microsomal subfractions The results indicate that the different microsomal membranes of the liver make up a system of close functional integration

Dallner G PROPERTIES OF THE ROUGH MICROSOMES

From a morphological point of view rough surfaced endoplasmic reticulum is a very homogenous orgnelle The purpose of this investigation was to find out whether the vesicles produced by the homogenization do or do not represent a homogenous population with identical properties and a similar role in membrane and enzyme synthesis Rough microsomes were isolated from rat liver with a discontinuous sucrose gradient system and this fraction suspended in 0 44 M sucrose was layered on a continuous sucrose gradient, extending from 20 to 55 per cent. Centrifugation at 60 000 g for one hour resulted in a sedimentation of 30 per cent of protein and 40 per cent was recovered from the upper third of the gradient The RNA/protein phospholipid/protein and cholesterol/phospholipid ratio did not exhibit significant differences The sedimentation coefficient varied between 0 4 × 10^3 and 1 2 × 10^3 s and the isopycnic equilibrium density between 1 17 and 1 25 Analysis of NADH and NADPH-cyt c red well as glucose 6 phosphatase activities gave an uneven distribution the specific activities of the top fractions significantly exceeding those in the pellet A pronounced enzyme synthesis occured following starvation pheno barbital treatment and (after birth) was seen also in the newborn For this reason the rough microsomes of such systems were also analysed The newly synthesized enzymes appeared first in the top fractions glucose 6 phosphatase after starvation NADPH-cyt c red after phenobarbital treatment and NADH ferricyanide and NADPH-cyt. c red in the newborn In adult and newborn rats glycerol ^3H incorporation was distributed evenly but was decreased in the top fractions of rough microsomes from phenobarbital treated rats 3 min after ip injection of leucine ^{14}C all subfractions in the adult rat contained a high radioactivity A stimulation of the incorporation rate occured after phenobarbital injection especially in the top

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fractions It also appeared that the microsomes from the upper part of the gradient were the most active in newborn rats

These results indicate that the rough microsomes of the liver are remarkably heterogenous The explanation of this heterogeneity can be at least twofold The first alternative is that a piece of the membrane is built up for a specific purpose the second that enzymes and perhaps also membranes are synthesized in one compartment and later transferred to another one

Ericsson J IN VIVO LABELLING OF CYTOSOMES FOR FINE STRUCTURAL STUDIES OF AUTO AND HETERO PHAGOCYTOSIS

Cytosomes are subcellular organelles showing histochemical activity of acid hydrolases and hence appear to represent "lysosomes Although cytosomes are readily recognizable in cells under normal conditions labelling of these organelles with electron opaque material appears to be important for their proper identification during interaction with other cytoplasmic structures in certain pathological conditions Exogenous compounds which are picked up by cells through endocytosis or otherwise and are resistant toward attack by lysosomal enzymes appear to be stored in cyto omes

Quantitative labelling of cytosomes with electron dense material was attained with the following methods 1) Intramuscular injections of Fe^{++} to rats or mice (iron sorbitol-citric acid complex Jectofer) 4 mg/100 gm of body weight for 10 consecutive days (labels cytosomes in renal proximal and distal tubules and collecting ducts hepatic parenchymal cells Kupffer cells and macrophages and certain secretory cells) 2) intravenous injections of thorium dioxide (thorotrast) (25 per cent solution 0 5 ml/100 gm of body weight) 3–5 injections on consecutive days (labels cytosomes in hepatic parenchymal cells Kupffer cells and macrophages)

Following induction of autophagic activity with hypoxia (rat hepatic parenchymal cells) or i p injection of bacitracin (mouse renal proximal tubule cells) forming and early cytosegresomes were separate from prelabelled cytosomes Later cytosegresomes contained label while ordinary labelled cytosomes were reduced in number or absent These observations suggest that cytosegresomes attain lysosomal enzymes by fusion with pre existing cytosome A similar fusion appeared to occur between endocytosis vacuoles in renal proximal tubules and prelabelled cytosomes

Brun A & Brunk U HISTOCHEMICAL STUDY OF HEAVY METALS IN THE RAT BRAIN AT VARIOUS AGES

Sprague Dawley rats of both sexes aged 3 and 8 weeks, and 9 12 and 18 months were used A brain slice containing the neocortex and hippocampus wa treated according to our modification of the sulphide silver method

The metals appeared as small and large granules in the cytopl m f ocortical nerve cells mic oglial and vascular endothelial cells and to some t nt n astrocytes but not in ligodendroglia or myelin A diffu e y l ow deep b n olouration was also seen n th nerve cell cytoplasm corti l n ur phi i hippocampal mossy fibre la r harply corresponding to the H –H area of Ro e A succe si e incre se of gr nul s and diffuse stain ng constantly a companied ageing indicat ng accumulation of l metals At all ages the neoco tical ne cells of l mina 5 were richest in l y m tals f llowed by lamina 2 and 3 6 nd 4.

The distributi appearance of the small and large granules strongly sug

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gested that the heavy metals were located in different types of lysosomes. The diffuse staining of the neuronal cytoplasm may be due to the presence of free heavy metals The accumulating free as well as the lysosomes bound fractions are likely to exert toxic effects on the ageing nerve cell

Borum Kirstine THE ABILITY OF REGENERATION OF THE MOUSE THYMUS AFTER PARTIAL SURGICAL THYMECTOMY

Lagerlöf B CYTOPHOTOMETRIC ANALYSIS OF THE DNA CONTENT IN X RAY INDUCED THYMOMAS

The DNA content in thymus cells of C57Bl mice under various experimental conditions has been analysed by means of cytophotometrical measurements on Feulgen stained smears. Thymus cells from normal untreated adult mice have very uniform DNA values and show a very sharp peak in the diploid region in the DNA histograms Regenerating thymus lobes from irradiated mice which failed to develop tumours have signs of slightly increased DNA synthesis In X ray induced thymomas harvested when very small and thus in a very early stage of development there was evidence of increased DNA synthesis but no indication of aneuploidy In the late disseminated thymomas harvested at the terminal stage of the disease the thymoma cells showed very active DNA synthesis with broadening of the diploid peak and many cells in S and G 2 phase In a spleen metastasis a large proportion of the cells seemed to be of tetraploid nature and in another two cases there was slight indication of aneuploidy No definite correlation between the histological degree of differentiation of the tumours and the pattern of the DNA histograms was found and there was not either any correlation between the mean and the deviations of the DNA values

Stenram U Nilsson Eskil & Berg Rebekka THE EFFECT OF SOME CANCER THERAPEUTIC AGENTS ON THE TRANSPLANTABILITY AND GROWTH OF AN SV 40 SARCOMA IN RAT

The effect of some cancertherapeutic agents on a previously described (Int J Cancer 1 139 1966 Acta path et microbiol scandinav in press) transplantable SV 40 induced rat sarcoma has been studied In culation of 1000 tumour cells produced progressively growing tumours in all control rats Rats that had been treated with cyclophosphamide or thiotepa did not develop progressively growing tumours The difference was statistically significant ($P < 0.01$) The effect of cyclophosphamide has also been studied on growing palpable tumours Cyclophosphamide initially retarded the tumour growth so that a statistically significant difference in tumour size was obtained ($P < 0.001$) However most of the tumours grew progressively also in the treated rats

Linell F THE MEMOIRS OF ROKITANSKY

Kjellgren O Eliasson C Lowhagen T Schnürer L B & Angström T PATHOLOGICAL AND CLINICAL MEANING OF THE GROUPS OF PAPANICOLAOU

Linell F & Roneus O SLIDE SEMINAR ON PARASITIC DISEASES IN MAN AND ANIMALS

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IMMUNOLOGY

Lundgren G Moller G & Zukoski Ch Laboratory of Tran plantation Immunology Department of Surgery Serafimerlasarettet Stockholm FFFECTS OF HUMAN GRANULOCYTES AND LYMPHOCYTES ON HUMAN FIBROBLASTS *IN VITRO*

Human fibroblast monolayers were initiated from skin biopsies Lymphoid cells and granulocytes were isolated from defibrinated blood by gelatin sedimentation 97–99 per cent pure lymphocyte suspensions were obtained by treatment with carbonyl iron powder which facilitated the removal of the granulocytes with a magnet 0 1 ml of the appropriate cell suspensions were pipetted onto the surface of the monolayer at indicated sites The destruction of the monolayer (plaque formation) was read after 1–6 days —5 $\times$ 10^5 granulocytes caused plaques within 4–8 hours whereas 10^4 cells required 96–144 hours for a detectable effect. Plaque formation was immunologically non specific since allogeneic and autochthonous target cells were affected to the same extent Phytohaemagglutinin (PHA) was not required Plaque formation by granulocytes was not abolished by treating the cells at 56 C for 30 minutes or by disintegrating them with ultrasound —Non immune pure *lymphocytes* only caused plaques in the presence of PHA and their effect was completely abolished by pre treating the cells at 48 C for 30 minutes or by ultrasound disintegration —The plaque forming ability of the granulocytes was probably due to a release of enzyme from the granules It was shown that the fibroblasts only detached from the surface of the petri dishes and remained viable in the medium since it was possible to recultivate them from the supernatant. In contrast plaques caused by PHA t eated lymphocytes revealed a true cytotoxic effect.—The studies emphasize the importance of separating completely the granulocytes from lymphocytes in studies of the cytotoxicity by lymphoid cells A granulocyte contamination of 1–5 per cent is sufficient to cause false positive results

Wasserm in J Lack ilen Th Perlmann P & Je l iann Hel j Ce t 1 Bacte io logical Lab ratory f St ckholm City and The Wenne G en I t u Stockholm CYTOTOXIC LYMPHOID CELLS AND ANTIBODIES FROM GUINEA PIGS IMMUNIZED WITH TUBERCLE BACILLI

Guinea pig were inje ted once with BCG v ccine and sub q ntly 7 times with killed tuber le b illi in paraffin oil Spleen cells r lymph ytes from the pe ripheral blo d f these animals were incul ated with ^{51}Cr labelled fowl erythrocytes coat d with PPD Immune lymphoid cells l s d the PPD c ated e throcytes within

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20 hours as assessed by measuring isotope release to the medium Serum from immunized animals contained an antibody which rendered lymphoid cells from normal guinea pigs cytotoxic in the same system Cytotoxicity of normal lymphoid cells was observed after preincubation of the PPD coated erythrocytes or of the lymphoid cells with immune serum Incubation of the cells in the presence of immune serum gave similar results. Guinea pigs lymphoid cells could be replaced by human rabbit or even autologous fowl lymphocytes but not by guinea pig kidney cells thymocytes or human Burkitt lymphoma cells Addition of complement was not required for these cytotoxic reactions which could be inhibited by means of rabbit anti guinea pig globulin serum or by addition of PPD in the medium

Björklund B & *Norén Birgitta* National Bacteriological Laboratory Stockholm
DISTRIBUTION OF CHARACTERISTIC ANTIGENICITY IN MALIGNANT AND NORMAL HUMAN TISSUES

In previous studies characteristic antigenicity could be attributed to a pool of human epithelial carcinomas as compared to a pool of normal human tissues (Int Arch Allergy 8 149 19 6 10 56 1957 10 153 1957) Immunochemical studies revealed that the pertinent tumour antigenicity was linked to lipoprotein structures (Int. Arch Allergy 17 241 1958) Cross reacting antigenicity was found in a variety of human carcinomas and HeLa cells (J Nat Cancer Inst 26 533 1961)

Previous studies had been performed by cytotoxicity inhibition technique Common antigens have now been observed also by other techniques such as haemagglutination haemagglutination inhibition and immuno diffusion inhibition A population of 42 human carcinomas was compared with population of 48 normal human tissues Anti HeLa serum was used to demonstrate the antigen within the tissue populations A statistically significant difference ($P < 0.001$) in distribution of the tumour antigen was found in the malignant tissues as compared to the normal ones These findings attest to the validity of the original concept of common antigens in a wide variety of carcinomas

Jonsson Jonas Biberfeld Gunnel & *Fagraeus Astrid* The National Bacteriological Laboratory Stockholm THE INCIDENCE OF AUTOANTIBODIES IN A GROUP OF APPARENTLY HEALTHY PEOPLE

The incidence of antibodies against thyroglobulin the second colloid antigen the surface and cytoplasm of thyroid epithelial cells against gastric parietal cells and against cell nuclei was investigated in 200 males and 184 females who were apparently healthy at the time when blood samples were obtained The sera were examined with the passive haemagglutination immunofluorescence complement fixation and mixed haemadsorption techniques At a low titre level ($\geq 1/10$) the incidence of antibodies against all the tissue components examined was relatively low (0–8 per cent) and about equal for males and females in the age group 20 40 years The incidence then increased with increasing age to 15 40 per cent for females and 7–20 per cent for males at 60 80 years of age The diagnostic significance of auto antibodies when found in low titres must therefore be evaluated with due regard to the age and sex of the patient According to our experience up to the present time the following average titre levels in a single serum sample may serve to differentiate between pathological and non pathological reactions

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Antibodies to	Method of examination	Titre level
Thyroglobulin	Passive haemagglutination	≥ 1/1600
Second colloid antigen	Immunofluorescence	≥ 1/10
Thyroid cell cytoplasm	Immunofluorescence	≥ 1/10
	Complement fixation	≥ 1/5
Cell nuclei	Immunofluorescence	≥ 1/10
Gastric parietal cells	Immunofluorescence	≥ 1/10

Britton S Dept of Bacteriology Karolinska Institutet Stockholm INDUCTION OF IMMUNOLOGICAL PARALYSIS *IN VIVO* AND *IN VITRO*

By treating phenolextracted lipopolysaccharide (the O antigen) of E coli 055 B5 with weak alkali it was possible to detoxify the molecule without obtaining any apparent changes in its antigenicity With this detoxified material the antigen dose *could be increased to the level where immunological paralysis was induced in* inbred mice The range between immunizing and paralysing dose of this antigen was found to be more narrow than that protein antigens. Since it was possible to study single antibody forming cells against this antigen using passively sensitized sheep red cells as targets in a modified agar plaque test of Jerne the cellular kinetics of tolerance induction in virgin and immune cell populations could be followed No significant cellular antibody formation could be observed before paralysis was expressed in a non immune animal If an immunized animal was given a paralytogenic dose of the immunizing antigen a constant lag period of 50–80 hours was observed before the paralysis was expressed at the cellular level This lag period which proved to be of the same order as that of antiserum mediated inhibition of the immune response in the same system indicates that antibody producing cells divide for a certain number of cell generations without further antigeneic stimulation

Furthermore it was shown that normal lymphoid cells treated with the same detoxified antigen *in vitro* (60 in 37) in the ratio 0 1–0 01 mg CI S/10[6] lymphoid cells were rendered unresponsive to an immunizing dose of the homologous antigen in a lethally irradiated syngeneic host This unresponsiveness was specific since cells treated in the same way responded normally to other antigens *e g* sheep red blood cells With extrinsically labelled antigen one could estimate that less than 0 2 per cent of the antigen remained on the cells after washing *in vitro* The results support the hypothesis that the direct contact between antigen and immunologically competent cells results in specific tolerance

Chesebro B Bloth B & Svehag S E Department of Immunology National Bacteriological Laboratory and Department of Virus Research Karolinska Institutet Stockholm THE ULTRASTRUCTURE OF NORMAL AND PATHOLOGICAL IgM IMMUNOGLOBULINS

Free IgM immunoglobulins were examined in the electron microscope using the negative contrast technique Normal human and rabbit IgM and Waldenstrom s macroglobulin were indistinguishable from one another and revealed flexible spider like particles with five appendages joining a central ring The average total span of the molecules was 300 Å The appendages were about 125 × 30 Å and the

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central ring had an outer diameter of approximately 100 Å and an inner diameter of 40 Å

Some purified 19S IgM preparations tended to form massive aggregates (> 50S) which when examined in the electron microscope revealed enormous clumps of IgM molecules whose appendages were entangled with one another

Electron microscopy of reduced alkylated IgM revealed total absence of intact spider like molecules The predominating structure observed was a round electron dense knob about 50 Å in diameter which in some cases had a fine fiber like extension with approximate dimensions 100 × 15 Å

Rabbit and human IgM molecules with antibody activity to poliovirus dried in sodium tungstosilicate on a carbon film as in preparation for electron microscopy were shown to retain nearly 100 per cent of their poliovirus neutralizing activity following redissolving in a physiological buffer

Karlsson Karl Axel & Thal E Research Institute of National Defence Sundbyberg and National Veterinary Institute Stockholm EXPERIMENTAL STUDIES BY MEANS OF FLUORESCENT ANTIBODIES (FA) IN ANTISERA OF YERSINIA PSEUDOTUBERCULOSIS

Globulin fractions from antisera of the 5 groups of *Yersinia pseudotuberculosis* (Y pstb) *Yersinia pestis* (EV 76) *Yersinia enterocolitica Salmonella typhimurium* and *Salmonella dublin* were conjugated with fluorescein isothiocyanate The FA reaction with O and OH sera of *Y pstb* was O group specific The serological relationship between O group II of *Y pstb* and *Salmonella* group B and between O group IV of Y pstb and *Salmonella* group D is reflected by the FA technique In OH sera also the specifity of the fluorescence is determined by O antibodies The quality of the FA conjugates however does not depend on the O agglutination titre No FA cross reactions between *Y pstb* and *Y pestis* occurred

Cnarpe H & Laurell G The Institute of Medical Microbiology Department of Bacteriology University of Uppsala Uppsala O GROUPING OF URINARY E. COLI WITH THE AID OF FLUORESCENT ANTIBODY TECHNIQUE

Coloform bacteria cause most of the urinary tract infections Recently it has been found that O groups 01 02 04 06 018 and 075 are responsible for nearly 50 per cent of the infections of the urinary tract In the present investigation the authors have performed O grouping with the aid of direct fluorescent antibody (FA) technique of coliform bacteria isolated from a selected paediatric material during the period from January to June 1967

In cases in which there was significant bacteriuria i e more than 10 bacteria per ml O grouping in 32 out of 72 specimens was possible Where the bacteriuria was not considered significant i e less than 10^5 bacteria per ml O grouping was possible in 36 out of 128 specimens

The clinical material was analysed In 9 out of 14 patients considered to have infections of the urinary tract the bacterial strains were groupable 90 patients were considered as noninfected and O grouping was possible in 19 of these

With FA staining of slides prepared from centrifuged urine the authors could find and group the bacteria in 6 urine specimens from 6 patients later considered to be suffering from urinary tract infections among 19 patients who were con

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sidered to be noninfected this was possible only in three cases Fluorescent bacteria were verified by conventional culture and tube agglutination test

Holmgren J Eggertsen G Hansson Lars A Jedal U Lincoln K & Winberg J
Institute of Medical Microbiology and Department of Pediatrics University of Goteborg Sweden THE ANTIBODY RESPONSE OF CHILDREN WITH PYELONEPHRITIS TO DIFFERENT ANTIGENS OF *E COLI*

Using immuno diffusion methods complex antigenic patterns could be demonstrated in strains of *E coli* of the O groups most frequently found in patients with urinary tract infections More than 20 antigenic components were observed in various antigen preparations such as veronal buffer extracts freeze pressed bacteria and ultrasonically disintegrated bacteria O antigens prepared according to Westphal contained 2–4 precipitinogens They only precipitated with their homologous O antisera with only one exception the earlier known cross reaction between the O antigens of coli 018 and coli 04 This specificity favours the assumption that one or more of the components in these Westphal preparations correspond to the O antigen

Sera from infants and children with first time and recurrent pyelonephritis were studied with the double diffusion method using various antigen preparations of the infecting *E coli* strains Precipitating antibodies against the O antigens as well as against several other coli antigens were found in sera from the patients with recurrent infections No such antibodies have been observed in preliminary studies of sera from children with their first infection and from blood donors These precipitating coli antibodies have been localized in the 7S fraction after gel filtration of the patient sera The antibody titres of the sera obtained with passive haemagglutination did not relate to the presence of precipitating antibodies The haemagglutination titres found after reduction of the sera with 2 mercaptoethanol presumably given by IgG antibodies showed a good correlation to the amounts of precipitating antibodies apparent from the immuno diffusion analyses

This study shows that the immuno diffusion methods are useful in the analysis of the antibody response to the infecting *E coli* strain in children with pyelonephritis By revealing the presence of antibodies to several different coli antigens these methods add to the results obtained with the usually used passive haemagglutination technique In addition the precipitation method demonstrates mainly the 7S antibodies whereas the passive haemagglutination technique favours the 19S antibodies.

VIROLOGY AND CELL BIOLOGY

Norrby E Department of Virology Karolinska Institutet Stockholm
STRUCTURAL ANTIGENS OF ADENOVIRUS TYPE 3 To be published in Virology 1968

Wadell G Norrby E & Skaar I P Department of Virology Karolinska Institutet Stockholm THE SOLUBLE HAEMAGGLUTININS OF ADENOVIRUSES BELONGING TO ROSEN'S SUBGROUP III 1 THE RAPIDLY SEDIMENTING HAEMAGGLUTININ Submitted for publication in J Gen Virology

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Pettersson Ulf Philipson Lennart & Höglund S Division of Cellbiology The Wallenberg Laboratory and The Institute of Biochemistry Uppsala
PURIFICATION AND CHARACTERIZATION OF HEXON AND FIBRE ANTIGEN FROM ADENOVIRUS TYPE 2

Gelfiltration on 4 per cent agarose was used as a method for partial purification of the structural proteins of adenovirus type 2 The hexon antigen was further purified by DEAE chromatography preparative polyacrylamide electrophoresis and sucrose gradient centrifugation

The fibre was extensively purified by DEAE chromatography isoelectric separation and gelfiltration on 6 per cent agarose

The final products were homogenous by analytical ultracentrifugation polyacrylamide electrophoresis electronmicroscopy and immunoelectrophoresis The hexon has a calculated molecular weight of 400 000 and the fibre 60 000 The pure hexon contains only one antigenic determinant which appears to be groupspecific Neutralizing antibodies could not be induced when purified hexon was injected into rabbits Electronmicroscopy showed that the hexon is a complicated structure with a central whole and probably six subunits. Six fold symmetry of the hexon could be demonstrated with the stroboscopic technique The fibre appears as a rodlike structure with a spherical unit at one end Amino acid analysis of the hexon revealed a low content of arginine as compared to intact virus which speaks in favour of an internal component rich in this aminoacid

Lycke E & Ronn O Department of Virology Institute of Medical Microbiology University of Goteborg Goteborg Sweden
STUDIES ON NON GENETIC REACTIVATION OF VACCINIA VIRUS

Öberg B & Philipson Lennart Division of Cellbiology The Wallenberg Laboratory University of Uppsala Uppsala Sweden
REPLICATION OF POLIOVIRUS RNA STUDIED BY GELFILTRATION

Gelfiltration on sphere condensed agarose (Sepharose®) has been shown to be a convenient method to separate single and double stranded poliovirus RNA The rate of P^{32} labelling of single stranded viral RNA is ten times higher than that of double stranded RNA Labelled double strands are synthesized early in infection Extrapolation of the ratio single to double stranded RNA to the addition of P^{32} indicates that double stranded RNA is synthesized prior to single strands

Less than 5 per cent free minus strands was recovered after a P^{32} labelling for 1–2.5 hours post infection less than 1 per cent when labelling from 1–3 hours and less than 0.1 per cent when labelling from 1–5 hours post infection The parental genome could not be detected in double stranded viral RNA after infection with high activities of P^{32} labelled poliovirus Chasing with cold phosphate for 3.5 hours after P^{32} labelling for 1–3 hours post infection did not result in a decrease of the label in the plus strands of the double stranded complex Infectivity of single stranded RNA from purified virus and double stranded RNA purified by gel filtration was 6.0×10^5 PFU/μg for single stranded and 6.3×10^4 PFU/μg for double stranded RNA when determined on monolayers in the presence of DEAE dextran

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Heden C G & Molin O Bact Bioeng., Bacteriological Department, Karolinska Institutet Stockholm LARGE SCALE CULTIVATION OF HUMAN DIPLOID CELLS ON TITANIUM DISCS

An apparatus has been developed which can be adapted for large scale cultivation of cells growing in monolayer The cells are grown on rotating titanium discs in a 1000 ml culture vessel The discs could be exposed to varying centrifugal forces while in the culture vessel This makes it possible to selectively harvest cells Titanium was found to present an excellent culture surface Cells attached rapidly and the growth rate and maximal cell densities were comparable or better than tho e obtained on glass or plastic surfaces

The apparatus has many advantages for instance a large surface to volume ratio (a maximum of about ?000 cm per 500 ml medium) Rotating the discs also en sures good mixing of the medium and an efficient aeration The design further provides means for an easy separation of the tissue culture cells from the medium

BACTERIOLOGY

Lofstrom G Hallander H O & Laurell C Department of Medical Microbiology Division of Bacteriology University of Uppsala Uppsala Sweden
IDENTIFICATION OF STAPHYLOCOCCAL HAEMOLYSIN STIMULATED BY PENICILLIN IN LOW CONCENTRATIONS

Attempts we e made to identify the haemolysin which was produced when cer tain strains of staphylococci grew in the presence of low concentrations of peni cillin G The concentration of 0 03 IU of the antibiotic in broth was used and the haemolysin (0 03) was compared with haemolysin (0) produced without stimulation by penicillin It was already shown in a previous paper (1) that two active frac tions appeared by separation in a Sephadex G 100 column There was evidence that the 0 03 haemolysin eluted in a second peak, which also was the peak containing α lysin In Sephadex G 25 the haemolysin eluted at the void volume and using Sephadex G 50 the haemolysin appeared in a fraction immediately after the void volume These results indicated that the haemolytic substance in question had a molecular size which was of the same order as ordinary α lysin This lysin has a molecular weight of about 40 000

In immunoelectrophoresis purified α lysin could be identified against anti α lysin as a welldefined precipitation band

The haemolytic substance of a 0 03 culture was found to occur in a definitely *higher* concentration in comparison with a O product in a precipitation line which showed identity with a α lysin band

It was al o found that anti O serum could fully neutralize the haemolytic ac tivity of the O lysin as well as that of the 0 03 lysin

Ord nary α lysin (O lysin) is destroyed by heat (100 f r 1 m nute) and also destroyed by trypsin (37 for 30 min) The 0 03 lysin ha l the same cl aracteristics

It was considered that the evidence indicated that the p o ti n of haemolysin stimul ted by penicillin consisted of an in rea e in the pr lu t n of α lysin There was however also evidence that a cert s le t   n c e of various other staphylococcal enzymes occurred Examinati   n p re   n these questions.

Reference

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Lincoln K & Lidin-Jansson Gunilla Institute of Medical Microbiology Department of Clinical Bacteriology University of Goteborg Goteborg EFFECT ON THE FAECAL COLI FLORA OF ORAL SULPHONAMIDE TREATMENT

About 20 children with diagnosed febrile urinary tract infection were treated in hospital with Cantrasin® 200 mg/kg/day for 10 days Samples of the rectal content were taken with anal swabs and cultured aerobically on blood agar and Drigalski agar 6–10 *E coli* colonies were selected from each culture and subjected to sensitivity testing and to O grouping with eight *E coli* O antisera (O1 O2 O4 O6 O7 O8 O18 and O75)

Before treatment an *E coli* strain of identical sensitivity pattern and O group as that of the infecting urinary strain was found to dominate the faecal flora in 6 out of 10 infants aged 3–6 months In another 3 infants such a strain could be isolated in small numbers only In only one case was it not possible to find the urinary strain in the faecal culture with the method used In most cases these primary strains were sulphonamide sensitive

During sulphonamide treatment there was in few days a remarkable change of the faecal flora The sensitive *E coli* strains disappeared and were replaced by sulphonamide resistant coli strains and enterococci From the fourth to the tenth day of treatment 91–98 per cent of all selected faecal *E coli* strains were sulphonamide resistant in a group of seven infants from which 51 anal swabs were cultured during a period of two weeks

One of these infants a boy of three months presented sulphonamide sensitive *E coli* O2 in the urine and in the faecal flora On the second day of treatment this strain had been replaced by sulphonamide resistant coli O4 and O18 The coli O4 strain dominated the faecal cultures of day 3 4 6 7 and 8 One week after cessation of therapy the boy was returned to the hospital with fever and with about 1 million sulphonamide resistant *E coli* O4 per ml of urine He was then treated in four periods with nitrofurantoin but 4 months after the initial sulfonamide treatment his faecal flora was still dominated by sulphonamide resistant *E coli*

The rapid development and sometimes long persistence of sulphonamide resistance of the faecal flora caused by oral sulphonamide in large doses offers a good explanation of the very high frequency in children of sulphonamide resistant recurrences of urinary tract infection occurring after an initially very succesful sulphonamide treatment of the primary infection as recently reported by *Bergström & Lincoln* (Acta Paediat Scand in press)

Liljedahl S O & Wickman Kristina Clinic of Surgery and Central Laboratory of Clinical Bacteriology Karolinska Sjukhuset Stockholm BACTERIAL GROWTH AND ANTIBIOTIC SENSITIVITY IN EXTENSIVE BURNS OBSERVATIONS FROM A FURTHER FIVE YEAR PERIOD

During the years 1954–58 385 patients with extensive burns were studied 170 of these having third degree burns over 15 per cent or more of the body surface Among 314 patients treated during 1962–66 211 had third degree burns over 15 per cent or more of the body surface During this period the burns were treated by the open exposure method and protein loss was compensated fully and early Antibiotics were given according to a principle of utmost restriction

The mortality rate was significantly lower during the second period namely 49 deaths as opposed to an expected 105 Hospitalization time for extensive burn cases was shortened During the first period 40 per cent of the deaths were caused

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by septicemia as against 30 per cent in the second period The frequency of severe broncho pneumonia was reduced fr m 20 per cent to 10 per cent

The bacterial flora was the same during both periods but reduced in amount during the sec nd The number of specimens without growth of pathogenic bacteria increased even from patients who were treated in the ward for longer than a week There was no increase in evere infections caused by gram negative reds Detailed sensitivity tests performed throughout the patients stay in ho pital showed that most of the bacteria found in the wounds during the second period had a higher sensitivity to most antibiotics

During 1966 when continuous treatment of extensive burns with warm dry air was started these favourable tendencies were even more noticeable

Unger P & *Wallmark G* Blood Tr nsfusion Centre and Bacteriological Laboratory So lersjukhuset Stockholm A VACUUM SYSTEM FOR TAKING BLOOD FOR CULTURE AND SEROLOGY

Blood culture is important for the detection and adequate treatment of septicaemia Blood is often drawn with a sterile syringe and transferred to a bottle with an anticoagulant This method involves a considerable risk of contaminating the blood To reduce this risk and to simplify the collection of blood we have suggested a closed vaccuum system which is now available as Vac ampin It consists of a sterile vacuum bottle w th an anticoagulant added connected by means of a short plastic tubing with a annula The taking of blood with this equipment is simple as is the handling at the laboratory It can be stored and thus always be at hand The sterility seems to be reliable

Altogether 563 blood cultures on blood taken with this sy tem were reported the blood being cultured in a solid and 3 liquid media 71 per cent showed no growth and 11 per cent significant growth of a variety of pathogenic species In 18 per cent one or a few colonies were observed all prob bly l boratory contaminations

A variant of the system with a vacuum tube instead of bottle is suitable for taking blood samples for serology chemistry bl od pre ervation by means of freezing tagging of cells by means of isotopes etc The tube is sterile and is thus also suitable for samples to be transported

Malmborg Anna Stina Department of Clinical Bacteriology Karolinska Sjukhuset, Stockholm A NEW PENICILLIN ACTIVE AGAINST PSEUDOMONAS AERUGINOSA

A new semisynthetic penicillin carbenicillin (BRL 2064) is active against certain strains of P eudomonas aeruginosa (A red et al Nature 215 5 1967) 32 patients suffering from m nifest infections of the urinary tra t due to Pseud monas aeruginosa have been treated with carbenicillin intram s larly for 10 20 days Quantitative urine ultures have been performed daily h n the t eatment and in a post treatment follow up The P eudomona t t ped before and after treatment All patients suffered fron l u r r tract in fections f r example kidn y or blad l r t t l l lasms or bladder paralysis due to para legia 14 patient h l ll t r

In 4 patients P eudomon s v s elim r th u ng treatment and the urine remained sterile luring th f e t t days In 11 patients P eudomonas was elimin t l but lur t l w up l they were reinfected with other bacteria uch s E ol l l t a d Proteus. In 13 patients

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the urine was temporarily sterilized but Pseudomonas reappeared in the urine after the cessation of treatment Phage typing showed that the majority of the relapses was due to the original phage type In 4 patients Pseudomonas was not eliminated during the treatment.

The favourable clinical results obtained indicate that carbenicillin is useful in the treatment of urinary tract infections caused by Pseudomonas aeruginosa

Tunevall G & Frisk A R Central Bacteriological Laboratory and Medical Service Ersta Hospital Stockholm POTASSIUM 6 (D α AZIDOPHENYL ACETAMIDO) PENICILLINATE, A NEW PENICILLIN[1]

The new compound in the following called azidopenicillin was tested with parallel serial dilution and disk tests against 89 recently isolated bacterial strains. It was found that zone diameters could be interpreted into terms of minimum inhibiting concentration according to the same regression as given by H Ericsson for penicillin G in 1954

The effect of azidopenicillin did not differ significantly from that of penicillin G or ampicillin whereas penicillin V most used as oral penicillin was less active than ampicillin against H influenzae In a larger material 4 5 strains tested only with disk method a better effect was found against enterococci for azidopenicillin than for penicillin G In all other respects these two compounds behaved similarly

In cross over experiments in 18 convalescent patients the serum concentrations given by an oral dose of 0 3 g azidopenicillin were compared with those following the same dose of penicillin V One hour after this dose the mean value for azido penicillin was 2 63 ± 0 25 as against 1 67 ± 0 17 for penicillin V After four hours the values were 0 21 ± 0 02 as against 0 06 ± 0 01 These differences are highly significant In another series with 14 patients 0 6 g azidopenicillin gave almost twice as high levels as the 0 3 g dose Considering its good activity and favourable pharmacological properties the new substance seems promising

Wallmark G Ost C R & Sallmander U Sodersjukhuset Stockholm BACTERIURIA AMONG WOMEN APPEARING FOR HEALTH EXAMINATION

In the course of a programme of health examination among employees of the city of Stockholm 1665 women at the age of 40 to 69 years were studied by means of quantitative urinary culture The second portion of a clean voided specimen immediately chilled was cultured Eighty 4 8 per cent had significant bacteriuria i e more than 10 bacteria per ml in two specimen E. coli and coliforms predominated (97 per cent) The counts were in general above 10^6 Almost all negative cases had a count below 10^4 Fifty five of the bacteriuric women were studied more carefully All but one had one or several episodes of urinary tract infection and 28 had slight symptoms at the time of study Intravenous urography was performed on 51 and six cases of previously unknown pyelonephritis was detected

Griess nitrite test (Stat test®) was compared to quantitative culture Provided that the patients had kept the urine in the bladder at least 5 hours before the test the reaction was positive in 84 per cent among 160 urines with a count of 10^5 or more False positive reaction was observed in 0 1 percent

[1] The penicillins used in this study was placed at our disposal by AB Astra Sodertalje

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It is concluded that the detection of bacteriuria in women is important and should be included as a routine in health examination programmes The becteriuric cases should be treated and followed up by means of new cultures

Larje O & Frostell G Institute for oral microbiology Karolinska Institutet Stockholm ACID PRODUCTION ACTIVITIES OF CARIESINDUCING STREPTOCOCCI

Some years ago it was demonstrated at the National Institutes of Health Bethesda Md USA that dental caries in rodents could be induced by specific streptococci These streptococci produce polysaccharides out of sucrose and are very important components of dental plaque The mentioned micro organisms are not to be found in Bergey s manual.

Since acid production by plaque micro organisms is generally considered to be a predominant cause of apatite dissolution in dental caries it was considered of interest to study the acid production capacity of caries inducing streptococci in comparison to that of non-cariesinducing streptococci

Acid production in suspensions from carriesinducing streptococci was studied by means of an automatic titrator From dextrose fructose and sucrose was noted the production of acid of the magnitude 100–200 × 10⁹ E/ mg d w/min From lactose sorbitol and hydrogenated potatostarch hydrolysate the acid production was very low

Acid production from related but non cariesinducing streptococci was also studied The acid production performed by these streptococci was of the same magnitude as that of the cariesinducing streptococci

The rate of acid production performed by a certain kind of filaments (Odontomyces viscosus) was also very low These micro organisms had been shown to form dental plaque but not induce caries in rodents

This study supports the theory that a cariesinducing micro organism must be able to form dental plaque as well as producing a great amount of acid.

Myrback K E Ringert O & Dahlstrand S National Bacteriological Laboratory Stockholm AND EPIDEMIC OF TULARAEMIA IN SWEDEN DURING THE SUMMER OF 1967

The winter epidemic of tularaemia in northern Sweden declined during February 1967 The latest cases occurred in early May

In the end of June new cases of human tularaemia were reported—this time from the county of Norrbotten When this new epidemic began to decline by the end of August 1199 cases had been reported in the northern part of the country and among these 1308 occurred in the county of Norrbotten

This outbreak of tularaemia was more dissem nate l th n an other hitherto known in Sweden and it started about three we ks e r tl n earlier summer epidemics

The majority of cases sho ed tl e ul er gl d l r t p f tulara mia and the route of infection was in most instan es m squ t a a f transmis ion well kno n from earlier summerepidemi n (

The agedistribution of cases sho ed o k t d scre n y to th t of the population There was no overrep esentation) y e up tion

A serological survev sh wed an infection ate that as lower than that seen in

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the airborne winterepidemic The incidence of infected individuals without clinical symptoms was extremely low 3 7 per cent

To explain the early appearance and the magnitude of the epidemic the following points can be mentioned. Voles and hares were known to be infected by F tularensis in an unusual high rate The animaltribe had not been reduced as much in the county of Norrbotten as in the county of Jämtland The hot and long summer made optimal conditions as to density and survival of the mosquitos

Ringert O & Dahlstrand S National Bacteriological Laboratory Stockholm

CULTURE OF *F TULARENSIS* IN THE 1966–67 OUTBREAKS OF TULARAEMIA IN SWEDEN
LABORATORY METHODS AND PRECAUTIONS AGAINST LABORATORY INFECTIONS

During the two outbreaks of tularaemia in Sweden in 1966 67 culture for *F tularensis* was carried out in total on more than 200 samples mostly spleen and liver tissue from hares or voles The material was suspended in saline and injected intraperitoneally into guinea pigs The guinea pigs died within 3–21 days and material from liver and spleen was transferred to a culture medium containing tryptose broth cystein HCl sodiumthioglycolate glucose and rabbit blood (*Gaspar* 1961) F tularensis bacteria were isolated from 34 out of 107 hares and 18 out of 49 samples from voles Positive cultures were also obtained from vole faeces collected on snow outside a barn from contaminated hay and from 4 out of 7 specimens from human tularaemic ulcers

The laboratory work was carried out in a separately ventilated laboratory Protective clothing gloves mouth shields etc were used Most infectious work was done in a safety cabinet

The personnel was vaccinated against tularaemia with a live vaccine received from U S Army Biological Laboratories Fort Detrick Local reaction was obtained in 92 per cent of the vaccinated subjects and general symptoms mostly headache and fatigue was reported by 25 per cent Agglutination titres were found in 94 per cent No cases of laboratory infection occurred in the vaccinated personnel while 4 cases occurred among workers in a veterinary laboratory before the vaccination had been carried out there

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CONGENITAL VALVULAR AORTIC STENOSIS

Histological Changes in the Valves and Myocardium in 3 Cases

By

ARNE SERCK HANSSEN[1]

Received 6 xi 67

Aortic stenosis is one of the more common congenital malformations in the heart *Abbot* (1936) found 23 cases amongst 1000 autopsies *Campbell & Kauntze* (1953) found nearly 3 per cent amongst their cases of congenital heart disease and *Nadas* (1963) 5 per cent amongst his cases

Although subaortic and supra aortic localizations of the stenosis occur *Nadas* (1963) states that the valvular type represents close to 75 per cent *Peckhorn Keith & Evans* (1964) found amongst 83 cases 64 valvular and 19 subvalvular stenoses

The increasing frequency of surgical correction of all types of congenital heart disease including aortic stenosis makes it important to have a full understanding not only of the primary pathology of the heart but also of the secondary changes

The purpose of this communication is to present the morphological changes in the valves and myocardium in 3 cases of congenital aortic valvular stenoses A comparative histological study has been made of the malformed valve and the foetal aortic valve with the purpose of gaining an understanding of the genesis of the malformation The morphological findings are related to a functional study obtained by cardiac catheterization of one of the cases (case 3) 5 days prior to death

CASE REPORTS

Clinical Extracts

Case 1 R F Born 23/11 62 Died 8/1 63 Autop y no 4 63 Health parents Spontaneous delivery 6 weeks before estimated te m Weight at b th 0[illegible]0 gram The day after b rth slight cyanosis was noted and s toli m r wa hea d Angiocardiograph through the umbilical ve n re ealed that tl right side of the heart was normal Investigation of the left side was unsatisf tory for technical reasons Hi c t gradually improved and he was discharge n De mb r 13th only to be re l one week later be au e of respiratory li t e pallor and anoxia The t l nurmus had greatly increa ed in intensity and there was a palpable thrill l nically considerable enlargement of the h art particularly to

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the right of the sternum He was treated with digitalis with considerable improvement but on January 8th he was found dead in bed

Case 2 S H h Born 11/11 63 Died 16/12 63 Autopsy no 1630/63) Healthy parents. Spontaneous delivery at term Nothing wrong was noted at the maternity hospital From 3/12 63 symptoms of pyloric stenosis appeared and he was treated with Eumydrin drops On december 15th he was admitted to hospital because of respiratory distress He was very ill with symptoms of shock and the respiratory distress increased and terminated in death the following day without a diagnosis having reached

Case 3 C B Born 2/2 64 Died 13/? 64 Autopsy no 2?3/64 Healthy parents Spontaneous delivery at term appeared normal for the first 3 days After that there was gradually increasing cyanosis and a systolic murmur was heard He was admitted to the children s department On February 14th a cardiac catherization was performed revealing a considerable left to right shunt at atrial level together with a very high pulmonary arterial pressure (Table 1) On February 19th while being fed the child vomited aspirated and died in the course of a few minute

TABLE 1

Results of Cardiac Catheterization in Case 3

	V cava inf	V cava sup	R atrium	L atrium	L vent	R vent	Pulm Art
O_2 in %	68 8	41 6	86 6	96 2		84 7	8? 7
Pressure in mm Hg			11 ?	13½	98/6½	97/9½	100/47

Autopsy Findings

The changes in the hearts were essentially the same in all 3 cases Heart and body weights were

Case 1 Heart 48 grams Body 2860 grams
Case 2 Heart 56 grams Body 37?0 grams
Case 3 Heart 5? grams Body 3?70 gram

All cases had a severe aortic stenosis due to greatly thickened nodular valves The nodules were smooth and shining (Figs 1 and 3) projecting into the aortic orifice whilst the sinus of Valsalva was free The greatly thickened valves occupied at a rough estimate from ½ to ⅔ of the cross sectional area of the aortic outlet (Figs ? 6 and 7) All cases had bicuspid valves

All cases had a marked left ventricular hypertrophy and a considerable hypertrophy of the right ventricle as well

The cut surface of the myocardium had large pale fibrous looking areas particularly in the posterior part of the interventricular septum The left anterior papillary muscle of the mitral valves had a light brown to yellow colour in all cases (Fig 2)

The endocardium of the left ventricle was slightly thickened in all cases especially in the subaortic region

None of the cases had anatomical closure of the foramen ovale and in Case 3 a sickle shaped defect at the free margin of the septum pri



Figs 1–2

Fig 1 Nodular thickened aortic valve (arrow) and left ventricular hypertrophy From Case 1
Fig 2 Left anterior papillary muscle revealing pallor due to necrosis extending to its base From Case 2



Fig 3
Aortic orifice viewed from above revealing greatly thickened bicuspid valve and and origin of coronary vessels (arrows) From Case 3

mum was convincingly demonstrated while the left atrium was still undisturbed and distended by blood (Fig 4)

Ductus Botalli was closed in Case 1 by a combination of thickened intima and thrombosis whilst no anatomical closure was effected in Cases 2 and 3

The coronary arteries of all cases were normal with a normal origin (Fig 3) No malformations were noted in any other organs

Microscopy

The valves The base of the aorta which had been opened longitudinally in Cases 1 and 2 but left intact in Case 3 was removed with the valves Sections were made in a plane transverse to the direction of the



Fig 4
Greatly dilated right atrium Arrow points at sickle shaped defect at margin of septum primum The hypertrophy of the right ventricle is evident From Case 3

blood flow Sections were stained with H & E alcian green alcian blue PAS and Verhoeff s elastic tissue stain All valves revealed essentially the same changes *i e* a greatly increased amount of primitive mesenchymal tissue partly in a nodular fashion interposed between the endothelial layers of the valves (Figs 5 6 and 7) The primitive mesenchymal tissue had a loose myxomatous ground substance that stained light pink with H & E light green with alcian green distinct blue with alcian blue and faintly positive with PAS The cells embedded in the ground substance were rather small spindle or star shaped of the type usually seen in myxomatous tissue (Figs 12 13 and 14)

Small omounts of fibrin were occasionally seen in crypts on the surface of the valves There were no inflammatory changes or vascularization In elastic tissue stain some elastic fibres were seen at the attachment to the aorta but no fibres were seen in the bulk of the thickened valves (Fig 6)

The myocardium Sections of the myocardium and papillary muscles were stained with H & E Van Gieson s and Mallory s stain for collagen Von Kossa s stain for calcium and Verhoeff s elastic tissue stain Sections from all cases revealed essentially the same changes *i e* extensive interstitial fibrosis with well formed wavy bands of collagen (Fig 8) areas of marked perivascular fibrosis (Fig 9) and extensive coagulation necrosis in the core of the papillary muscles (Fig 10) In Cases 2 and 3 areas of calcification and fibrosis were also seen in the papillary muscles No inflammatory reaction was seen anywhere

The endocardium The endocardium in the subaortic region was slightly thickened with some elastic fibres penetrating a few millimeters into the myocardium

DISCUSSION

In these 3 infant boys who died 46 35 and 17 days old respectively the following morphological changes were found in the heart

1) Valvular aortic stenosis with marked general cardiac hyper trophy
2) Extensive myocardial fibrosis
3) Ischaemic necrosis of the anterior papillary muscle of the left ventricle
4) Slight endocardial thickening in the subaortic region of the left ventricle

Angiocardiography was performed only in 1 case (No 3) This revealed

A) Left to right shunt at atrial level
B) Pulmonary hypertension of severe degree

Incidence

It is evident from the literature that there must exist a wide spectrum of severity of the condition from cases that present with symptoms of cardiac insufficiency at or shortly after birth to cases presumably not giving symptoms until secondary changes such as calcification occur in adult life The exact incidence is therefore very difficult to ascertain

During the 5 year period 1960–1964 32 774 children were born in the City of Oslo In 3 of these congenital valvular aortic stenosis was the cause of death in early infancy One further child is attending the cardiac clinic under this diagnosis Thus provided there is no accumulation of cases in this 5 year period approximately 1 child out of 8 000 appears to be born with severe or at least early symptomatic valvular stenosis

It has been impossible to obtain any reliable figures regarding the incidence of latent cases It is possible however that a substantial part of the cases of aortic stenosis seen in adults without a proven rheumatic aetiology may have developed on the basis of congenitally thickened valves This view is also expressed by *Campbell & Kauntze* (1953)

The Valvular Aortic Stenosis (Figs 5–7 11–14)

Although considerable attention has been paid to the various macroscopic types of valvular aortic stenosis (*Gould* 1960 pp 408–409) little is written about the microscopic appearance and the possible pathogenesis of the condition

The bulk of the greatly thickened valve is made up of primitive embryonal connective tissue avascular and without inflammatory reac



Figs 5, 6, 7

Transverse sections of the aorta Cases 1, 2 and 3 respectively Greatly thickened nodular bicuspid valves Alcian blue Verhoeff's elastic stain and Alcian green × 12

…ion (Figs 12–14) The microscopic findings therefore do not support an inflammatory genesis The histology and staining properties of this tissue are very similar to those of the primitive endocardial cushions of the foetus (Fig 11)



Normally an invagination or excavation takes place from the distal surface of the cushions with the formation of the sinus of Valsalva (*Hamilton Boyd & Mossman* 1962) and the layer of loose connective tissue is thinned out and achieves the dense structure of mature connective tissue In the case of valvular aortic stenosis there is a persistence and probably also continued growth of the embryonal connective tissue The aetiology is unknown

The Myocardial Fibrosis (Figs 8 9)

Extensive fibrous and degenerative changes in the myocardium of a 3 day old infant who died with severe aortic stenosis have been described (*Brown* 1934) Otherwise the myocardium is rarely commented on or has been described as normal (*DuShane* 1954)

In the presented cases the young age of the patients and the far advanced fibrosis with mature collagen fibres make it probable that the fibrosis at least in part must have been established during uterine life In the absence of any evidence of inflammatory changes the only likely cause of the fibrosis is an intrauterine myocardial hypoxia This may be explained by the aortic stenosis Normally during foetal life oxygenated blood reaches the left atrium through the foramen ovale This trans septal circulation is conditioned by a lower pressure in the

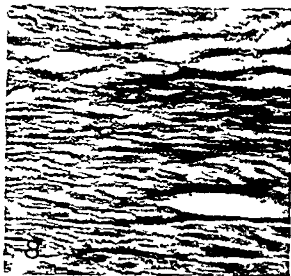


Fig 8
Myocardium from Case 2 revealing diffuse interstitial fibrosis Van Gieson's stain for collagen × 225

left than in the right atrium secondary to the small pulmonary circulation

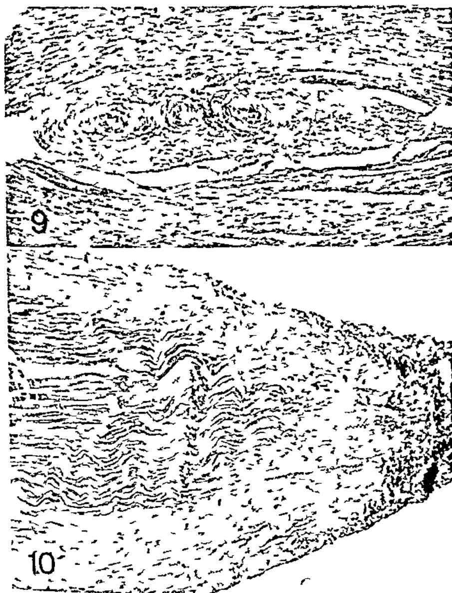
If the aortic stenosis is sufficiently marked to reduce the output from the left side of the heart the pressure in the left atrium will increase and the admixture of oxygenated blood will be correspondingly reduced Thus the blood flowing through the coronary arteries will be less than normally oxygenated

Other possible explanations also applying to the period after birth are a) The suction effect on the coronary ostia by the high velocity flow through the small aortic opening b) the squeezing effect on the coronary arteries by a high ventricular systolic pressure and c) a shortened diastole (due to tachycardia)

The Papillary Muscle Necrosis (Figs 2 10)

The necrosis of the central part of the anterior papillary muscle in the left ventricle can only be explained by conditions aggravating the mechanisms referred to above

The left anterior papillary muscle normally derives its blood supply from the left anterior descending branch of the left coronary artery and lies at the greatest distance from the ostium of the artery (*Gross* 1921) A fall in the coronary circulation pressure would lead to the most serious consequences in this distant area of supply In adults the left anterior papillary muscle is the favourite site of scarring probably for the same reason (*Monckeberg* 1924)



Figs 9–10

Fig 9 Myocardium from Case 1 revealing marked perivascular fibrosis H&E × 85
Fig 10 Papillary muscle from Case 3 revealing extensive coagulation necrosis in central part H+E × 35

Endocardial Thickening

Left ventricular endocardial thickening associated with congenital aortic stenosis is well known At times the endocardial thickening involves the whole left ventricle and is so marked that the ventricle as

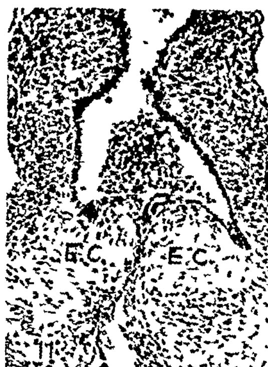


Fig 11

Longitudinal section through the endocardial cushions (E.C.) and ascending aorta of a 18 mm human foetus The nucleated red cells are seen just above the primitive valve H&E × 225

sumes the appearance seen in diffuse endocardial fibroelastosis This is probably the reason why it has been suggested that the aortic stenosis is secondary to the fibroelastosis and is a result of fibro elastic tissue growing into the aortic valves

In the cases presented here the aortic stenosis is very marked The endocardial thickening however is slight and histologically there is no evidence of fibroelastic tissue from the endocardium extending to the aortic valves It is felt therefore that the endocardial thickening is secondary to the aortic stenosis and probably the result of a combination of increased left ventricular pressure and anoxia Anoxia as a cause of endocardial fibroelastosis has been described by *Johnson* (1952) in a study of 210 cases with congenitally malformed hearts

Left to Right Shunt at Atrial Levels (Fig 4 and Table 1)

In Case 3 the oxygen saturation values of blood obtained by cardiac catheterisation revealed a large communication at atrial level Morphologically an insufficient closure of the ostium secundum was noted This defect was judged to be due to a dilatation of the atria with a



Figs 12 13 14
Sections from the central parts of the thickened aortic valves in Cases 1 2 and 3 respectively The similarity to the histology of the foetal endocardial cushions is evident H+E. × 225

considerable increase in the diameter of the ostium secundum with a resultant defect in the valvular closure by the septum primum It is possible however that the defect may have been caused by under development of the septum secundum although this is considered less likely A similar defect in the valve of the foramen ovale in association with infantile aortic stenosis has been described by *DuShane* A left to right shunt was presumed to have existed although cardiac catheterization was not performed

The great similarity of the 3 presented cases both with regard to the degree of aortic stenosis and the secondary changes in the heart makes it probable that a left to right atrial shunt had existed also in Cases 1 and 2 although no note was made at the time of autopsy of an anatomical defect Such a relative defect may easily be overlooked however if the examination of the atrial septum is not undertaken while the left atrium is distended with blood

Pulmonary Hypertension and Right Ventricular i
(Fig 4 and Table 1)

Although a considerable pulmonary hypertensi cardiac catheterization only in Case 3 all cases h hypertrophy of the right ventricle It is probable tha 1 and 2 also had a severe pulmonary hypertension i that the hypertrophy of the right ventricle developed p life and partly after birth The pathogenesis however, mu in the two periods

In accordance with the above statements an abnormally l of blood reaching the right atrium must have passed int ventricle during foetal life The work performed by the vent have been increased but not as a result of increased pressu foetal pulmonary vessels have a considerable reserve capacity *Wegelius* 1954) and the ductus arteriosus is open

After birth it is fair to assume that the failing left ventricle increased pulmonary pressure with secondary right ventricular h trophy The pulmonary artery pressure in Case 3 (Table 1) sugg that it would be an advantage if the ductus arteriosus remained fu tionally patent If so the foetal direction of the flow from the pulm nary artery to the aorta would be maintained In Case 1 the ductu arteriosus was closed by a thrombus and it is not impossible that this was the cause of the rather sudden deterioration in the child s condi tion 3 weeks before death

SUMMARY

Three infants boys with congenital valvular aortic stenosis died at ages ranging between 17 and 46 days (after birth)

The histology of the deformed valves is compared with the histology of the foetal endocardial cushions and it is concluded that the stenosis probably results from persistence and continued growth of the embryonic connective tissue of the cushions

In addition to the valvular deformity a marked fibrosis of the myocardium and a necrosis of the anterior papillary muscle of the left ventricle was found The myocardial fibrosis is believed to have developed partly during intrauterine life because of hypoxia due to reduced right to left shunt through the foramen ovale The necrosis of the left anterior papillary muscle is explained on the same basis with the additional effect of the remoteness of this part of the myocardium from the ostium of the coronary artery

In one case cardiac catheterization 5 days prior to death revealed a left to right shunt at atrial level This shunt is believed to be secondary to a dilatation of both atria leading to an increased diameter of the ostium secundum

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SPONTANEOUS ADRENOCORTICAL LIPID DEPLETION IN MICE

Relationship to General Growth, Degeneration of Adrenal X Zone and Maturation of Seminiferous Epithelium

By

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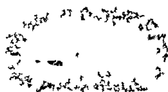
In the AKR/O strain of mice and in the hybrid lines CS and AC, derived from this strain, *Arnesen* and his co-workers have described a spontaneously occurring lipid depletion of the adrenal cortex, which is due to the action of a single recessive gene *ald* (*Arnesen* 1955, 1956, 1958, 1963, 1964 a and b, 1966, *Molbert & Arnesen* 1960, see also *Green* 1966). Figures 1 and 2 show Sudan III stained frozen sections of adrenals from adult males of the AC substrain and the C57Bl strain, the latter exhibiting a normal pattern of adrenocortical lipids.

The AKR/O strain has a high incidence of spontaneous leukaemia. From hybridization experiments carried out between the AKR/O strain and a non-leukaemic strain, WLO, the two different hybrid substrains CS and AC have emerged. Both substrains demonstrate the same inherited adrenocortical lipid depletion as the AKR/O strain. The substrain CS has an incidence of lymphatic leukaemia of about 60–70 per cent, whereas the AC strain is free from leukaemia (*Arnesen* 1964 b).

Earlier investigations have shown that the adrenocortical lipid depletion takes place between 30 days and 12 weeks of age, and probably coincides with sexual maturation (*Arnesen* 1956). Fig. 3 shows a Sudan III stained section of an adrenal gland from a prepubertal AC male mouse, 21 days old. Mice with spontaneous adrenocortical lipid depletion, however, have not been systematically studied with a view to defining the interrelation of loss of adrenocortical lipids, age of the animals, their body weight, and the signs of their sexual maturation. In the present paper such investigations will be reported.

The first part is a study of the adrenocortical lipid pattern of male AC mice at ages ranging between 1 and 80 days. It is shown that the adrenocortical lipids are rapidly lost when the animals are between 30 and 50 days old, which roughly corresponds to the period when the adrenal X zone degeneration is known to take place in male mice from other strains. Due to the coincidence in time, the adrenal X zone de-





Figs 1–3

Fig 1 Adrenal gland of AC male mouse 60 days old body weight 23 0 grams Spontaneous adrenocortical lipid depletion Sudan III × 33

Fig 2 Adrenal gland of C57Bl male mouse 81 days old body weight 27 0 grams Normal adrenocortical lipid pattern Sudan III × 33

Fig 3 Adrenal gland of AC male mouse 21 days old body weight 6 0 grams Normal adrenocortical lipid pattern Sudan III × 33

generation in male mice has been related to sexual maturation No systematic correlative studies however have been undertaken to test this hypothesis In the second part accordingly the lipid depletion of the adrenal cortex is studied in relation to the X zone degeneration as well as to the maturation of the seminiferous epithelium in male AC mice 30 to 50 days old

MATERIAL AND METHODS

The material consists of 2 series of AC males The animals were kept under standard conditions as described elsewhere (*Molne K* In prep) The animals were taken at random from the cages They were weighed to the nearest 0 5 g and killed by fracturing the neck The organs to be examined were quickly dissected free and fixed as described below

Series 1 Adrenals from 80 AC male mice 1–80 days old were studied 5 animal for each 5 days interval The adrenals were fixed in 4 per cent neutral formaldehyde (Baker s formaldehyde) for 2 days embedded in gelatine and cut on the freezing microtome with the microtome set at 15 microns The sections were stained with the Sudan III method of Daddi (*Romeis* 1948) the nuclear staining being omitted Micro photographs were taken with a Zeiss photomicroscope on Agfa Isopan IIF Ian chromatic film (15 din) using filters to obtain maximum contrast On the negatives the image of periadrenal fat was retouched to escape disturbing factors during the evaluation Contact copies were made from all the negatives in one performance The copies were signed by code numbers The senior member of the staff (Arnesen) then ranged the copies blindly into 7 classes according to the relative content of lipids The scale units 7–5 were interpreted as representing variations of a normal adreno cortical lipid pattern and the units 3–1 variations of the state of lipid depletion The results are expressed in Tables 1 2 and Fig 4

Series 2 Adrenals and testes from 30 AC males were studied The animals were 20–49 days old one animal for each day The organs were fixed in Bouin s fluid for 1 day dehydrated and cleared in tetrahydrofuran and embedded in fibrowax Sections were made with the microtome set at 3 microns and stained with haematoxylin eosin The sections of the adrenal gland and the testes were evaluated blindly and separately and classified into 3 degrees of development according to the following criteria

Adrenal X Zone

Immature state Fully preserved X zone without any sign of degeneration (Fig 5a)
Transitional state Diminuition of the width of the X zone signs of degeneration of the X zone cells no medullar capsule cells (Fig 5b)
Adult state X zone completely disappeared medullar capsule established (Fig 5c)

Testis

Immature state Solid cords of cells no tubule formation no spermatids or spermatozoa present (Fig 6a)
Transitional state Maturating seminiferous epithelium Some cords transformed into tubules Spermatids and even a few spermatozoa present but picture of the fully mature testis not established (Fig 6b)
Adult state Distinct tubules uniformly present seminiferous epithelium mature distribution and differentiation of cells characteristic of adult age (Fig 6c)

The results obtained independently by the 2 investigators were compared without breaking the code Four adrenals out of 30 were differently interpreted with respect to the state of the X zone by the two investigators These cases were discussed without breaking the code and a final decision according to the rank grading was made The ranking of all testis sections was consistent Thereafter the code was broken The results are expressed in Tables 3 and 4

RESULTS

The Relationship between Body Weight and Age in Young AC Males

The observations are presented in Table 1 and Fig. 4. The body weight increases following a slightly curved line probably a parabel. The growth has apparently not come to an end at the age of 80 days in spite of the fact that sexual maturity has been reached before this age. Pregnancies resulting from brother sister matings in the eight and ninth week of age are common in AC mice.

TABLE 1

The Relationship between Age and Body Weight in 80 Male AC Mice
The Figures Indicate the Number of Animals within Each Age and Weight Group

Body weight in grams	Age in days							
	1–9	10–19	20–29	30–39	40–49	50–59	60–69	70–79
1 3	5	–	–	–	–	–	–	–
4–6	4	3	3	–	–	–	–	–
7–9	1	4	1	1	–	–	–	–
10–12	–	3	5	1	–	–	–	–
13 15	–	1	1	6	2	5	–	–
16–18	–	–	–	2	5	3	1	–
19–21	–	–	–	–	3	5	7	2
22–24	–	–	–	–	–		2	8

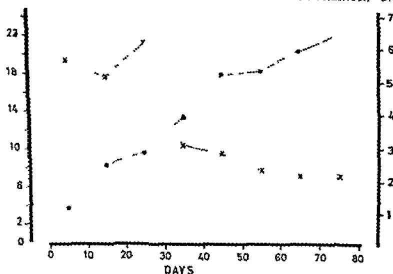


Fig. 4

The relationship between age and body weight ( ) and the relationship between age and adrenocortical lipid content (x) in young AC male mice

The Change in Adrenocortical Lipid Pattern of Young AC Males

The data obtained are demonstrated in Table 2 and Fig 4 It seems convenient to study the total age interval 1–80 days divided into 3 periods Mice younger than 30 days those between 30 and 50 days and those between 50 and 80 days

TABLE 2

The Relationship between Age and Adrenocortical Lipid Content Each Age Group Contains 10 Animals The Adrenals were Classified into 7 Groups According to the Lipid Content the Figures Indicate the Number of Animals within each Group Groups 7–5 Are Considered to Represent Variations of a Normal Adrenocortical Lipid Pattern Groups 3–1 Variations of Lipid Depletion

Lipid content in A L	Age in days							
	1–9	10–19	20–29	30–39	40–49	50–59	60–69	70–79
7	3	2	5	0	0	0	0	0
6	3 } 8	3 } 7	3 } 9	2 } 2	0 } 2	0 } 0	0 } 1	0 } 0
5	2	2	1	0	2	0	1	0
4	1 } 1	1 } 1	1 } 1	2 } 2	1 } 1	1 } 1	1 } 1	0 } 0
3	1	2	0	2	2	4	1	3
2	0 } 1	0 } 2	0 } 0	1 } 6	3 } 7	2 } 9	2 } 8	5 } 10
1	0	0	0	3	2	3	5	2

Twenty four out of 30 animals younger than 30 days had a normal adrenocortical lipid content (scale units 7–5) 3 were in a transitional state (scale unit 4) and 3 showed the slightest degree of lipid depletion according to our ranking system The greatest variation in Sudanophilia is evident in mice at ages between 30 and 50 days and it appears that the reduction of cortical lipids mainly takes place in this period Further the decrement of lipids seems to start rather abruptly in the age group 30 to 39 days In the age group 50 to 80 days only one out of 30 males was judged to have a normal adrenocortical lipid content 2 were in a transitional state and 27 out of 30 animals showed lipid depletion according to the criteria adapted

The X Zone Degeneration in Young AC Males

The results are given in detail in Table 3 and summarized in Table 4 From the age of 37 days the adrenal X zone had disappeared in all animals In mice younger than 29 days the X zone was present In the interval between 29 and 37 days there was a great variation in respect to the status of this zone In the majority of the animals younger than 37 days the X zone degeneration was judged to have started It should be emphasized however that the early phases of the X zone degeneration in many cases are hard to asses There are few cytological signs of degeneration and the diagnosis must be based upon the width of the



Fig 5a Adrenal gland of AC male mouse 21 days old body weight 6.0 grams Immature state fully developed X zone (arrow) H+E × 92

Fig 5b Adrenal gland of AC male mouse 32 days old body weight 10.5 grams Transitional state Degenerating X zone (arrow) H+E × 92

Fig 5c Adrenal gland of AC male mouse 49 days old body weight 20.5 grams Adult state The X zone has disappeared peri-medullary capsule cells are seen (arrow) H+E × 32

actual cortical zone From Table 3 it appears that the X zone degeneration may be somewhat better correlated to body weight than to age

TABLE 3

Age Body Weight and State of the Adrenal X Zone and Seminiferous Epithelium of 30 AC Males Arranged According to Increasing Age (One Animal for Each Day)

Age (Days)	Body weight (Grams)	Adrenal X zone	Semiferous epithelium
20	5.0	I	I
21	6.0	I	I
22	7.5	I	T
23	7.0	T	I
24	6.5	T	T
25	8.5	T	I
26	5.5	T	I
27	8.0	T	T
28	10.5	T	T
29	14.0	A	A
30	8.0	T	T
31	14.5	A	A
32	10.5	T	T
33	11.5	A	A
34	16.5	A	A
35	11.0	T	T
36	16.0	T	A
37–49	16.5–20.5	A	A

I = Immature state T = Transitional state A = Adult state

The Maturation of the Seminiferous Epithelium in Young AC Males

The results are presented in detail in Table 3 and summarized in Table 4

TABLE 4

Correlation between the State of the Adrenal X Zone and the Seminiferous Epithelium in 30 AC Mice 20–49 Days of Age The Material is subdivided into 3 Age Groups The Figures indicate the number of Animals

Adrenal X zone	Seminiferous epithelium		
	Immature state	Transitional state	Adult state
	10 animals 20–29 days of age		
Immature state	2	1	–
Transitional state	3	3	1
Adult state	–	–	–
	10 animals 30–39 days of age		
Immature state	–	–	–
Transitional state	–	3	1
Adult state	–	–	6
	10 animals 40–49 days of age		
Immature state	–	–	–
Transitional state	–	–	–
Adult state	–	–	10



Fig 6a Testis of AC male mouse 21 days old body weight 6.0 grams *Immature state No spermatozoas are seen* H+L × 221
Fig 6b Testis of AC male mouse 32 days old body weight 10.5 grams Transitional maturating state A few immature spermatozoas are seen H+L × 221
Fig 6c Testis of AC male mouse 49 days old body weight 20.5 grams Adult state Number and distribution of cells characteristic of adult age H+L × 221

From the age of 36 days all animals had an adult type of seminiferous epithelium. Individuals younger than 22 days were immature in respect to this parameter. Between 22 and 36 days of age the males showed varying degrees of testicular development. Again the maturation seems better correlated to body weight than to age.

The Correlation between the Adrenal X Zone Degeneration and the Maturation of the Seminiferous Epithelium

The results are given in Tables 3 and 4. Sections of the adrenal cortex and the testes from each animal had separately been classified into one out of 3 classes according to the degree of maturation. In 25 out of 30 cases the degree of development was the same. In 5 animals it differed but only by one grade. Among the 5 cases no systematic trend in the variation could be figured out. In one case the seminiferous epithelium was more mature, in 4 cases it was less mature as compared with the state of development of the adrenal cortex according to the criteria adapted.

Thus in AC males the adrenal X zone degeneration coincides with the histological maturation of the seminiferous epithelium.

DISCUSSION

Methods. The adrenocortical lipid pattern as defined by the Sudan staining can hardly be characterized in absolute units in a conventional system of measurement but it may well be referred to an arbitrary range scale. This kind of semiquantitation proved valuable in an earlier study of mice with spontaneous adrenocortical lipid depletion (*Arnesen* 1956). The method used in the present study is a slight modification of the former. Microphotographs of the Sudan stained sections were blindly evaluated by an unbiassed observer. Since the specimens passed through multiple steps in the histological and photographic processing, there are several sources of error. However, the procedures were standardized as described previously in this paper and possible methodological errors should be randomly distributed.

The paraffin sections of the adrenal glands and the testes were also evaluated blindly according to certain criteria characterizing the prepubertal, maturating and adult structure of these organs. The histological signs of the prepubertal and the adult state in these organs are clear cut. The transitional state is rather vaguely defined since it represents a series of successive stages of development. This might be better characterized in more specialized studies. However, for the present purpose a further subdivision seemed unnecessary.

The adrenocortical lipid depletion. Arnesen (1956) stated that in AKR/O mice at the age of 30 days the adrenocortical lipid content started to be reduced. In twelve week old mice the lipid depletion was

complete The interval between the former and the latter stage had not been systematically investigated

In the present study the degree of adrenocortical Sudanophilia of male AC mice 1–80 days of age has been studied The majority of mice younger than 30 days has a normal adrenocortical lipid content Between 30 and 40 days a rapid depletion of lipids occurs and in animals older than 50 days the adrenal cortex is generally lipid depleted It is noteworthy that during the period of rapid decrement of adrenocortical lipids (30–50 days of age) the weight gain curve does not indicate a *stressed situation (Table 1 and Fig 4)*

The literature dealing with the adrenocortical lipid pattern of young *normal* mice is sparse To our knowledge there is only one report on this subject *Whitehead* (1933) studied the adrenocortical lipid content of mice of various colours He found that in male mice 15–17 days old the lipids filled the entire permanent cortex Twenty eight days old animals had a lipoid band which occupied the outer 8/10 of the permanent cortex In males which were 37 days or older the relative width of the lipoid band was about 6/10 *Thus from the third to the eighth week* of postnatal life a certain reduction in adrenocortical lipids generally takes place in male mice Within this age period the adrenocortical lipid depletion takes place in AC mice The possibility therefore exists that the adrenocortical lipid depletion of these mice represents an extreme variant within a normal range of distribution However in the adrenal cortex of adult AKR/O hybrids the Sudan stainable lipid droplets are few and irregulary distributed and no lipoid band of continous *sudanophilic material is present (Fig 1) This picture resembles* the adrenal from a severely stressed animal

The degeneration of the adrenal X zone The first part of the present study showed that the onset of the adrenocortical lipid depletion of AC males takes place when the animals are at ages between 30 and 40 days This coincides roughly with the period of adrenal X zone degeneration in male mice (*Howard Miller* 1927 *Deanesly* 1928 *Waring* 1935 *McPhail & Read* 1942 *Chester Jones* 1948) According to these authors the degeneration starts at the age of about 4 weeks and is complete when the animals are 5–6 weeks old In the present study it is demonstrated that in some AC males the involution had started at the age of 23 days *and in all individuals older than 36 days the X zone had completely* disappeared Consequently in respect to the age period of adrenal X zone degeneration the AC mice seem to behave like other mouse strain

The maturation of the seminiferous epithelium There are few reports concerning the histology of the testis in maturating mice *Yocom* (1917) found that in 3 week old mice there were no spermatids or spermatozoa In 5 week old mice a few spermatozoa were found but apparently they were not fully mature being firmly attached to Sertoli cells *Parkes* (1925) made smears from the epididymis of young mice and correlated the findings to sections of the testis In cases where the

testis showed evidence of maturity active *spermatozoa* were also found in the smears. In animals older than 40 days the seminiferous epithelium showed an adult pattern. In our study of AC males the histology of seminiferous tubules was considered adult from the age of 36 days. Consequently our results are roughly consistent with the reports referred to.

It is noteworthy that the maturation of the seminiferous epithelium and the X zone degeneration probably represent *early* steps in the process of sexual maturation. *Howard Miller* (1927) found that when the adrenal X zone degeneration took place the *vesicula seminalis* had not reached the adult size. *Baillie* (1961) stated that the differentiation of the secondary sex organs in male mice is not fulfilled until the eight week of postnatal life. In AC mice the age of the first fertile mating has not been systematically investigated but pregnancies resulting from sister brother matings before the seventh week are very rare. Sexual maturity occurs about the same time in males and females or perhaps somewhat later in males (*Bronson, Dagg & Snell* 1966).

The relationship between the spontaneous adrenocortical lipid depletion and the sexual maturation. In this study the onset of the adrenocortical lipid depletion in male AC mice is shown to coincide with the adrenal X zone degeneration and the maturation of the seminiferous epithelium. *Arnesen* (1956) found that prepubertal gonadectomy prevents the occurrence of spontaneous adrenocortical lipid depletion. These results indicate that the spontaneous adrenocortical lipid depletion is a function of the complex process of sexual maturation.

In AC males the decrement of adrenocortical lipids may be explained as the result of the endocrine mechanisms involved in the maturation of the seminiferous epithelium and the adrenal X zone degeneration. The maturation of the seminiferous epithelium and the development of androgen producing cells is governed from the pituitary gland through different gonadotropic hormones. The X zone degeneration in male mice seems to be caused by androgenic hormones (*Deanesly & Parkes* 1937, *Chester Jones* 1949). Consequently the specific adrenocortical lipid pattern of AC mice may be related either to the function of the pituitary gland or to the endocrine function of the gonads.

These possibilities may be elucidated in hypophysectomy and gonadectomy experiments. Such investigations are in progress in our laboratory.

SUMMARY

Histological studies on male mice with spontaneous adrenocortical lipid depletion have shown that there is a correlation between the adrenocortical lipid content, the adrenal X zone degeneration and the maturation of the seminiferous epithelium.

Before puberty the adrenocortical lipid content is normal in all ani

mals In the majority of the animals the lipid depletion takes place when the animals are between 30 and 50 days old The adrenal X zone degeneration and the maturation of the seminiferous epithelium occur simultaneously and both processes appear to be completed at the age of 36–37 days Thus the onset of the adrenocortical lipid depletion coincides with the late stages of maturation of the testis and the adrenal X zone degeneration

The results are discussd with respect to possible connections between the spontaneous adrenocortical lipid depletion and the endocrine function of the gonads and the pituitary gland

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THE EFFECT OF HYPOPHYSECTOMY ON THE LIPID PATTERN OF THE ADRENAL CORTEX IN MICE WITH SPONTANEOUS ADRENOCORTICAL LIPID DEPLETION

A Histological Study

By

GEORG BRABRAND and KÅRE MOLNE

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Arnesen and his co workers have described a spontaneous lipid depletion of the adrenal cortex seen in adult mice of the AKR/O strain and of the hybrid substrains AC and CS derived from the latter it is determined by a single recessive gene the adrenocortical lipid depletion gene designated *ald* (see *Green* 1966) (*Arnesen* 1955 1956 1958 1963 1964 a and b 1966 *Molbert & Arnesen* 1960 *Molne & Brabrand* (in press))

Before puberty the cortical lipids are present in normal amount and distribution as judged by the staining with Sudan III At the time of sexual maturation the lipids disappear rapidly and in adult males the depletion is almost total (Figs 1 and 2)

The adrenocortical lipid depletion may reflect a primary abnormality of the adrenal cortex or it may be secondary to abnormal influences from other endocrine organs In this paper we shall present studies of the effect of the pituitary gland on the adrenocortical lipid pattern The possible effect of other endocrine organs (gonads see *Molne & Brabrand* in press) will not be considered here

Experiments with ACTH and cortisone have shown that lipid reaccumulation may take place in these adrenals in adult age suggesting that the spontaneous adrenocortical lipid depletion is related to the function of the pituitary gland (*Arnesen* 1956 1964 a) However the hormone preparations used in these experiments were not specific for mice and the doses were probably far outside the physiological range

Examination of the adrenals a short time after hypophysectomy

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We are indebted to dr *Jan Solem* who performed the determinations of the plasma corticosterone and to the head of the Life Insurance Companies Institute for Medical Statistics at the Oslo City Hospitals dr *Knut Westlund* for his helpful advice regarding the statistical analyses.

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tive but also qualitative differences between the adrenal cortex of normal animals and that of the AC strain in their responses to ACTH stimulation However it cannot be excluded that these differences may be due to the unspecificity of the ACTH preparation used or to inadequate dosage

The main conclusions to be drawn from these studies must be those drawn from the experiments in which ACTH substitution was omitted These experiments have clearly shown that a marked adrenocortical lipid re accumulation takes place in hypophysectomized mice with spontaneous adrenocortical lipid depletion In sham operated animals the adrenal cortex is further depleted Consequently the specific adrenocortical lipid pattern of these mice is dependent upon pituitary influence The results suggest that the pituitary adrenocortical relationship in normal animals and in mice in the AC strain differ but any further information concerning this difference is as yet not available

SUMMARY

Following hypophysectomy of male mice with spontaneous adrenocortical lipid depletion extensive lipid re accumulation takes place in the adrenal cortex In sham operated and in incompletely hypophysectomized animals the lipid depletion is even more extreme Post operative intensive ACTH treatment largely prevents the adrenocortical lipid repletion in completely hypophysectomized mice

It is concluded that the *spontaneous adrenocortical lipid depletion* is dependent upon pituitary influence

The results suggest an altered pituitary adrenocortical relationship in mice exhibiting this specific adrenocortical lipid pattern

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COMPARATIVE HISTOLOGICAL AND SPUTUMCYTOLOGICAL STUDIES OF THE BRONCHIAL EPITHELIUM IN INFLAMMATORY AND NEOPLASTIC LUNG DISEASE

By

MAGNUS NASIELL

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In previous studies (16 17 18 19) we have demonstrated a distinct connection between various alterations in the bronchial mucosa and lung cancer These alterations described in detail include bronchial metaplastic changes as well as certain abnormalities of the respiratory columnar epithelium The purpose of the present investigation was to study the occurrence and extent of these changes in cases with different types of chronic inflammatory lung diseases i e in tuberculosis and bronchiectasis and in cases with bronchogenic carcinoma using histological as well as cytological techniques Incidence figures of the alterations in various series are given taking differences in age and sex into consideration

Several investigators have found a relationship between metaplastic alterations and lung cancer and assume that bronchogenic carcinoma is preceded by such changes in the respiratory epithelium (1 3 12 15 25 34) Other authors found no support for this opinion (6 9 30) and some investigators consider metaplasia to be more strongly associated with other conditions than lung cancer (29) and particularly to chronic inflammatory lung disease (20 32 35 36)

The cytopathology of bronchial metaplasia based on studies of cytological material has only been discussed briefly (8 11 13 14) and incidence figures have not been included Reports on the possible course of bronchial metaplasia based on cytol[illegible] [illegible]d nce have also been few (21 28) One retrospective study (2[illegible] in[illegible]es some figures of histological and cytological chan[illegible]s [illegible] as metaplastic but not exhibiting an unmistakab[illegible] [illegible] metaplasia

Various abnormalities in resp[illegible] [illegible]olumn[illegible] [illegible]lls occurring in cytological specimens hav[illegible] b[illegible] [illegible] [illegible]tudies (11 13 14)

The author is indebte[illegible] t[illegible] D[illegible] [illegible] for [illegible]tatist[illegible] [illegible] analysis

Certain specific changes of ciliated respiratory cells called Ciliocytophthoria (CCP) by Papanicolaou have been described in greater detail These reports comprise incidence studies on the occurrence of CCP in pulmonary disease (22 23 27) The highest frequency of CCP has been shown to occur in patients with pulmonary carcinoma and in patients with pulmonary infections particularly in those of viral origin

MATERIAL AND METHODS

The *histological* material of cancer includes surgical specimens from 35 cases of bronchogenic squamous cell carcinoma 15 cases of bronchogenic undifferentiated carcinoma and 28 cases of primary pulmonary adenocarcinoma Surgical lung specimens from 91 patients with non specific and specific chronic inflammatory lung diseases have also been examined i e 34 cases with bronchiectasis lacking signs of active tuberculosis and 57 cases with active pulmonary tuberculosis 55 autopsy cases without primary lung cancer and lacking the inflammatory conditions mentioned above have served as control material 9 of these cases exhibited acute inflammatory lung changes In previous reports this control series included 56 autopsy cases (17 18) however one case with pulmonary tuberculosis was excluded now All cases were unselected except for the demand that the mean age in the control material should be similar to that of the cancer series This age distribution was not possible to attain in the groups with bronchiectasis and tuberculosis as the majority of these cases were surgical patients belonging in younger age groups (Table 2)

TABLE 1

Type of Specimens and Amount of Epithelium in the Histological Series

	Controls	Bronchiectasis	Tuberculosis	Adenocarcinoma	Squamous Undiff Ca
Total no of cases	55	34	57	28	55
No of cases with specimen					
Complete lung	55	3	5	20	43
Lobe	–	22	44	8	7
Segment	–	9	8	–	–
Average no of sections/case	9 5	13	9 5	11	12 6
Average no cm epithelium/case	19 8	29	16 4	24 3	18 2

The *cytological* material consists of 457 patients from different chest clinics These patients include 170 cases of lung cancer with a final clinical diagnosis of carcinoma including a histopathological diagnosis of the tumour type There were 90 cases with squamous cell carcinoma 47 cases with undifferentiated carcinoma and 33 cases with primary pulmonary *adenocarcinoma* The tuberculosis group is composed of 119 patients with a principal clinical *diagnosis* of active pulmonary tuberculosis The series of bronchiectasis comprises 26 patients with a principal clinical diagnosis of bronchiectasis without an element of active tuberculosis In an earlier report (18) a larger consecutive series of chest clinic patients without lung cancer was used as a control material This material included patients with various chronic pulmonary disorders All such cases have now been excluded and the remaining 142 patients who exhibit acute *inflammatory* lung diseases i e acute pneumonia bronchitis pleuritis (93 patients) or non respiratory diseases (49 patients) have been used as control series in the present study It had been preferable to use healthy individual

but this has not yet been possible because of the difficulty to obtain sputum from such cases. All carcinoma cases but 11 adenocarcinoma cases and all tuberculosis patients as well as 10 of the bronchiectasis patients are consecutive. The 14? control patients are also consecutive. The 11 cases with adenocarcinoma and 16 patients with bronchiectasis were chosen at random and were added in order to give larger cytological groups and thus more reliable frequency figures (Table 3).

Radiation treatment or cancer chemotherapeutic agents had not been given in any case before the sputum sampling or the histological preparation.

The type of specimen in the different *histological* materials is evident from Table I. This table shows that the majority of the bronchiectasis and tuberculosis cases were composed of lobectomy specimens and that the carcinoma cases mostly were composed of one complete lung as was also the case in all controls. In every case several sections were examined including whenever possible sections from main bronchi, lobe bronchi as well as peripheral bronchi. In all cases with complete lungs only one lung has been used. One third of the sections were taken from the main bronchi, one third from the lobe bronchi and the same number from the peripheral bronchi. In cases with lobectomy specimens one third to half the sections were taken from the lobe bronchi and the remaining sections from the peripheral bronchi. In segment specimens sections were always taken from the largest available bronchi. This study did not aim at indicating the exact location of the different changes in the bronchial mucosa but to point out the occurrence of the various alterations. The histological technique has been described in detail elsewhere (17). No sections from the immediate neighbourhood of the tumours have been included in this study.

The spread or extent of metaplastic changes was studied according to the number of sections per case exhibiting this alteration (Table 4).

In the *cytological* series all patients had at least one adequate sputum specimen, the majority had three or more, with an average of four smears prepared. All cytological specimens were stained according to the Papanicolaou technique. In this study sputum specimens were chosen as these represent a wider area of bronchial mucosa than secretion specimens obtained by bronchoscopy.

Statistics. In order to evaluate the frequency figures in the different material a χ^2 test (at a significance level of 0.5 and one degree of freedom) was used combined with the standardized population method. The following is a brief description of the procedure.

Some of the frequency differences in the compared materials could depend on different sex ratios or different age distributions. With the standardized population method the materials were divided with respect to sex and in 10 year age groups. Special weights were chosen for each of the intervals. A weighted mean was computed for each of the compared materials. If the weighted means differed from each other also a χ^2 test (two by two contingency table) with Yates correction for continuity was used to compute possible significance between the materials.

DEFINITION OF TERMS ✓

Slit formation in the bronchial epithelium means that there is a slit *between the columnar cells and the basal cells* (Fig. 1). The columnar cells are always altered above the cleavage and may show the picture of goblet cells or ciliated cells with different degrees of degenerative alterations. The intact adjacent epithelium do[illegible] not show these changes in the columnar cells (16, 17, 19).

Low epithelium without cilia [illegible] interpreted [illegible] the result of slit formation and expulsion of surfa[illegible] and [illegible] great similarity to the basal part of the epithelium [illegible] slit form[illegible] (16, 17, 19). The surface of low epithelium is sm[illegible] which [illegible]ishes it from an artefact where the surfac[illegible] (Fig. 2). It has lately been shown (10) that similar pict[illegible] the respiratory mucosa



Fig 3
Transitional metaplastic epithelium There is an increase in the number of basal cells with 2 rows of flattened cells on the surface No cilia are present. Case with pulmonary squamous cell carcinoma (v Gieson X 155)

and low epithelium as a result of exfoliation of degenerated surface cells can be produced experimentally

Metaplastic epithelium in the bronchial mucosa implies transition of the special cell type of this epithelium into the cell type of another epithelium In the bronchial mucosa this usually implies the occurrence of transitional epithelium and more rarely of a squamous epithelium instead of the ordinary ciliated epithelium (17) The cytomorphology of metaplastic cells of bronchial origin has been described in detail in a previous report (18) (Figs 3 and 4)

Atypical metaplasia Cellular abnormalities principally of the same type as in carcinoma but less pronounced may occur in the bronchial epithelium Such alterations occur almost only in metaplastic epithelium In a few cases some retained cilia were observed on the surface but the general appearance was that of metaplasia as described above In carcinoma in situ (non invasive carcinoma) the epithelium is composed [illegible] carcinoma like cells but [illegible] no submucosal invasion Such alterations were only seen in [illegible] the epithelium (Figs 5 and 6)

Micropapillomatosis This alteration which nearly always occurs in metaplastic epithelium implies the occurrence of stroma papillae in the bronchial epithelium exhibiting the appearance of stromal herniations into the mucosa The epithelium between the stromal herniations may also simulate cord like downgrowths as in irregular acanthosis (17) (Fig 7)

Abnormal columnar cell findings (ACCF) in cytological sputum



Fig 6

Group of metaplastic cells with nuclear abnormalities The nuclei vary in size and shape as well as in nuclear chromatin density Sputum Clinical diagnosis Pulmonary tuberculosis (Papanicolaou stain × [illegible]48)

specimens is the result of degeneration and destruction of large numbers of columnar cells in the bronchial epithelium This implies the occurrence of an abnormally large number of columnar cells in cytological specimens with non specific degenerative alterations (19) (Fig 8) as well as the occurrence of Papanicolaous CCP (Ciliocytophthoria) changes the latter changes include various characteristic alterations in the columnar cells such as fragmentation of cells occurrence of eosinophilic inclusion bodies and various chromatin abnormalities (22) (Fig 9) ACCF is interpreted as the sputumcytological result of slit formation in the bronchial mucosa which results in low epithelium (16 17 19)

RESULTS

The frequency figures for the epithelial changes in the different [illegible] are shown in Tables 2 and 3 Table 2 accounts for the histological materials and Table 3 for the cytological In both tables the [illegible] values are recorded only when a statistically significant difference between a certain group and the controls could be found The [illegible] age of the cases in the different series is shown in Table 2 and 3 Examination of the different frequency figures according [illegible] the standardized population method did not show any important [illegible] in age or sex in the material

It is evident from Table 2 that *slit formation* was most frequent in

the squamous undifferentiated (sq u) carcinoma series The incidence was significantly higher than that of the controls The incidence in the bronchiectasis and tuberculosis groups was also increased in comparison with the controls The difference became significant when the two inflammatory groups were combined (χ^2 = 4 2 for the combined group The obtained χ^2 value exceeds the critical value of χ^2 at a significant level of 05 and one degree of freedom) The adenocarcinoma series exhibited a similar incidence as the controls

Low epithelium was also most common in the sq u carcinoma material and least frequent in the control group The bronchiectasis and tuberculosis series exhibited almost as high values as the sq u carcinoma material the rate of all three groups was significantly higher than that of the controls while the rate of the adenocarcinoma group was only moderately elevated (Table 2)

The cytological phenomenon ACCF which seems to be related to slit formation and low epithelium showed the highest rate in the sq u carcinoma material and the lowest in the controls while the inflammatory and adenocarcinoma series took an intermediate position The tuberculosis adenocarcinoma and sq u carcinoma materials differ significantly from the controls (Table 3)

The incidence of *transitional or squamous metaplasia* was very high in the histological sq u carcinoma material (88 per cent) and significantly increased in relation to the frequency of the controls The incidence in the histological series with bronchiectasis and tuberculosis was on the same level as in the controls while that of the adenocarcinoma group held an intermediate position between these and the sq u carcinoma cases (Table 2) The frequency of metaplasia in the cytological sq u carcinoma series (80 per cent) was almost as high as in the corresponding histological group The lowest value was found in the control material (36 per cent) which showed a similar value as the histological controls (35 per cent) The cytological bronchiectasis and tuberculosis series and also the adenocarcinoma group exhibited moderately elevated values the rate in the tuberculosis as well as in the sq u carcinoma group was significantly increased (Table 3)

Figs 7–9

Fig 7 Microp ap llom tosis There re several pr t d ng from l p llae l h are c er d b a met plasti epithelium C f mall l nd ftc ent ated carcii r of the lung (× Cieson × 155)

Fig 8 Re p at column r cells with uncharacteri tic degen t v hanges The cyt pl n disintegrating and there are n c a The r l i var in size Sputum C s of lungtul ercul sis (P panicolaou stain × 300)

Fig 9 Colu nna c l in sputum v th pronounced leg n r t alt ratic n of the CCl type (l t re al ent Th nucl i exhibit pykn s and hromatin clump n One al eolar hi t ocyte in the left up p c ner Ca e of p dermo d l r ncho en c ca cinoma (Papan colaou sta n × 300)

TABLE

Incidence of the Histological

Total no of cases	Controls 55		Bronchiectasis 34		
Mean age	58 9		37 1		
	No cases	(%)	No cases	(%)	χ †
With slit formation	5	(9)	8	(20)	NS
With low epithelium	11	(20)	18	(53)	8 9
With metaplastic epithelium	19	(35)	9	(30)	NS
Of these cases with atypia	2	(4)	2	(6)	NS
With micropapillomatosis	13	(24)	2	(6)	NS

† The χ values are valid for the comparison with the controls and recorded only in con
NS = Not Significant compared with the control group

TABLE

Incidence of the Cytological

Total no of cases	Controls 142		Bronchiectasis 26		
Mean age	60 3		61 8		
	No cases	(%)	No cases	(%)	χ †
With ACCI	27	(19)	9	(40)	NS
With metaplastic cells	51	(36)	13	(50)	NS
Of these cases with atypia	33	(23)	7	(27)	NS

† The χ values are valid for the comparison with the controls and recorded only in con
NS = Not Significant compared with the control group

The incidence of *atypical metaplasia* was increased only in the squ carcinoma series the increase was statistically significant The other histological groups revealed similar rates All five cases with carcinoma in situ were found among the squ carcinoma cases The rate of atypical metaplasia in the cytological series was also significantly increased in the squ carcinoma material and only slightly elevated in the inflammatory and adenocarcinoma groups (Table 2 3)

The spread or extension of metaplastic changes in the bronchial epithelium as illustrated by the number of sections per case exhibiting this alteration is shown in Table 4 It is evident that the greatest extent was found in the squ carcinoma material in which 74 per cent of the cases showed metaplasia in more than one section and 46 per cent in more than three sections The corresponding figures in the adenocarcinoma group was considerably lower *i e* 39 per cent and 18 per

Changes in the Different Series

Tuberculosis 57			Adenocarcinoma 23			Squamous – Undiff Ca 50		
37 5			57 7			59 7		
No cases	(%)	χ †	No cases	(%)	χ †	No cases	(%)	χ †
14	(25)	NS	3	(10)	NS	16	(32)	7 2
29	(51)	10	10	(36)	NS	30	(60)	16
23	(40)	NS	1	(57)	NS	44	(88)	[illegible]9
4	(7)	NS	1	(4)	NS	25	(50)	27
3	(5)	NS	7	(30)	NS	30	(60)	13

nection with a significant difference at a significance level of 05 and one degree of freedom
Per cent of total no cases

3

Changes in the Different Series

Tuberculosis 119			Adenocarcinoma 33			Squamous – Undiff Ca 137		
55 2			64 5			63 5		
No case	(%)	χ †	No cases	(%)	χ †	No cases	(%)	χ †
49	(41)	14	13	(39)	5 [illegible]	76	(55)	38
60	(50)	5 0	16	(48)	NS	109	(80)	53
32	(27)	NS	11	(33)	NS	63	(46)	15

nection with a significant difference at a significance level of 05 and one degree of freedom
Per cent of total no cases

cent and in the controls still lower (20 per cent and 9 per cent) In the bronchiectasis and tuberculosis series the changes were most limited metaplasia in more than three sections was not found in any of these cases According to the same table these results are not influenced by the number of sections per case in the different groups

The squ u carcinoma series also exhibited the highest incidence of *micropapillomatosis* The difference in relation to the controls was statistically significant The adenocarcinoma group showed about the same frequency as the controls while th wo inflammatory series revealed a considerably lower incidence (*Table 2*) *In the control group* only 4 out of th 13 cases with micropapillomatosis did not exhibit a clear cut metaplastic epithelium (17) The connection between the two alterations is significant (χ = 7 2 which exceeds the critical value of χ^2 at a significance level of 05 and one degree of freedom)

TABLE 4
Extent of Metaplasia in the Histological Materials

	No of cases with meta plasia in >1 section/case	Average no of sections/case	No of cases with meta plasia in >3 sections/case	Average no of sections/case
Controls	11 (20%)	9.3	5 (9%)	8.8
Bronchiectasis	4 (12%)	10.3	–	–
Tuberculosis	7 (13%)	10.1	–	–
Adenocarcinoma	11 (39%)	13.0	5 (18%)	13.8
Squamous – Undifferentiated carcinoma	37 (74%)	10.1	23 (46%)	10.2

DISCUSSION

Slit formation in the bronchial epithelium is likely to be a fragile process which probably is easily destroyed during the histological preparation and hence difficult to preserve in the sections. This difficulty is also due to the fact that shortly after the completion of the epithelial cleavage the surface cells will be exfoliated. On the other hand low epithelium must be a more resistant and a longer existing alteration and thus easier to demonstrate in sections. This may explain the fact that the frequency of low epithelium in all histological groups is higher than that of slit formation (Table 2). ACCF has constantly been found in higher numbers in the cytological groups (Table 3) than slit formation in the histological. The reason may be that such loosened columnar cells are more easily caught in cytological specimens than in histological ones.

There appears to be a parallelism between the histological phenomena slit *formation—low epithelium* and the cytological ACCF in the different groups of the material. Thus the frequency is lowest in the controls and highest in the series with squ carcinoma. The two groups with chronic inflammation hold an intermediate position with respect to the histological as well as the cytological phenomena. Low epithelium however is almost as frequent in these groups as in the squ carcinoma series. It is of interest that the adenocarcinoma material does not show the same high incidence as the squ carcinoma group. Concerning ACCF the adenocarcinoma series is similar to the groups with chronic inflammation and with respect to the histological changes it is more similar to the controls.

It is reasonable to assume that this group of alterations is due to one or more factors which has an injuring effect on the respiratory epithelium particularly on its superficial cell layer. Repeated injury during a longer time consequently results in chronic inflammation. For that reason it is comprehensible that the frequency of changes is in

creased in the groups with chronic inflammation though the inflammation in these groups has been initiated not only by an injury to the superficial layer of the epithelium but also by a deeper penetrating injury to the tissue particularly by bacteriological infections The high incidence in the sq u carcinoma group indicates that a similar injury is an even more pronounced phenomenon in this group although the inflammatory reaction in the bronchial wall here is less marked than in the bronchiectasis—tuberculosis series On the other hand in adenocarcinoma the marks of injury of the surface cells are less prominent It was also repeatedly observed during this investigation that the inflammatory reaction in areas with metaplasia was not more pronounced than in areas with a normal epithelium In an earlier report (17) it has been pointed out that low epithelium which is the result of epithelial cleavage and surface cell expulsion during the subsequent repair may develop into a metaplastic epithelium and not into a normal respiratory epithelium

The high incidence of *metaplasia* in the histological as well as the cytological sq u carcinoma series shows that there is a strong and significant relationship between metaplastic changes in the bronchial epithelium and this type of lung cancer. It is of interest that the frequency figures are similar in the histological and cytological materials as to sq u carcinoma and controls In the different histological groups the metaplasia frequency is distinctly increased only in the sq u carcinoma series and only moderately in the adenocarcinoma group while the cytological material exhibits a distinct increase also in the tuberculosis material and a more moderate in the other groups This difference between the histological and cytological materials may be due to a greater possibility of cytology to detect metaplasia (18) The mean age that is higher in the cytological than in the histological inflammatory groups may on the other hand indicate that metaplasia is more common at a higher age however standardized population analysis did not show any importance of the differences in age

It has been a somewhat controversial question whether cells interpreted as metaplastic always originate from the bronchi or whether similar cells may originate from other regions i e nasopharynx To test this a material of 46 patients from all the cytological groups except the cancer groups was used These cases were chosen at random among patients exhibiting adequate as well as inadequate specimens and a presence of metaplastic cells in either the first adequate or in the first inadequate sample The definition of an adequate sputum sample is a specimen with alveolar macrophages In 42 cases metaplastic cells were found in the adequate specimen while one case exhibited such cells in the adequate as well as in the inadequate sample and three only in the inadequate sample

Admittedly this test is not quite decisive because it cannot be excluded that an adequate sputum specimen also may contain some

material from the upper respiratory tract The distinct association between the occurrence of metaplastic cells and adequate sputum samples indicates however that this source of error is not large

Atypical changes in metaplastic epithelium with cellular abnormalities similar to those in precancerous lesions (dysplasia and carcinoma in situ) of the uterine cervical epithelium were also found with *the highest frequency in the* squ carcinoma series This carcinoma group was the only histological group which showed elevated values 25 out of the 50 cases exhibited atypical metaplasia 5 out of these 25 cases revealed abnormalities consistent with carcinoma in situ—a picture only met with in this group In the cytological material there was some elevation of the values also in the other groups but a significant increase in relation to the controls was only found in the squ carcinoma series

Also with respect to metaplasia the adenocarcinoma group exhibits a lower incidence than the squ carcinoma series In the histological material the adenocarcinoma group shows a moderately higher metaplasia frequency than the groups with chronic inflammation In the cytological series the rate is similar to that of the inflammatory groups This is in agreement with the opinion that pulmonary adenocarcinoma may originate from the submucosal glands and not from the respiratory surface epithelium (7 25 26) Several studies of smokers show that pulmonary adenocarcinoma exhibits less dependence of extrinsic factors than squ carcinoma does (31) The higher incidence of metaplasia in the squ carcinoma series indicates that exogenous *factors are responsible for the development of metaplasia as well as* squ carcinoma

As the metaplastic alteration in the bronchial epithelium has to be considered as a basis for the development of squamous and undifferentiated carcinoma and the incidence of metaplasia is significantly increased in the cytologic tuberculosis group such inflammatory conditions may be of importance in carcinogenesis because of their metaplasia producing ability These circumstances support the assumption that chronic inflammatory lesions of the lung are of significance in the pathogenesis of pulmonary cancer (2 4 5 24 33 37)

It is however obvious that in cases where squamous or undifferentiated carcinoma has developed more effective factors than those of these chronic inflammatory conditions have produced an even higher *frequency of metaplasia Furthermore in this cancer group there is a* high incidence of cellular atypia in the bronchial mucosa

The incidence of atypical metaplasia is low in the non malignant histological groups but comparatively high (23–27 per cent) in the cytological This fact has been discussed earlier (18) One explanation may be that the cytological technique is more sensitive than the histological because it enables examination of cells from a wider area of bronchial mucosa A number of tests with representative slides from

our material have been performed in order to exclude overreading of cytological atypia in the presented material Thus our criteria of atypical metaplasia have been checked with those set up by several independent pathologists with different professional background In the evaluation it has constantly been found that atypical metaplasia is more frequent than non atypical

The extent of the metaplastic changes in the bronchial epithelium as evaluated by the number of sections per case exhibiting this lesion revealed that the extension of metaplasia in non cancer materials is inferior to that in cancer materials (Table 4) The lower extent in the two series with inflammatory disease as compared with the controls *may be explained by the lower mean age of patients in the former* groups The great extension of metaplastic changes in the squ carcinoma material may indicate that not only the occurrence but also the degree of extension of these alterations is of importance for the development of bronchogenic carcinoma It seems also possible that the degree of extension is dependent upon the duration of the factor which produces metaplasia as the inflammatory groups with their lower mean age exhibit such a limited extent of metaplasia

The squ carcinoma group also exhibited the highest number of cases with *micropapillomatosis* in the bronchial mucosa A significant *relationship between this cancer group and micropapillomatosis* was established This was not the case in the other series (Table 2) The controls and the adenocarcinoma cases showed a similar incidence while the figures in the bronchiectasis and tuberculosis series were considerably lower The lower mean age in these latter groups may be an explanation for this condition *i e* the occurrence of micropapillomatosis seems to be related not only to a particular tumour type but also to the age of the patients This assumption is not possible to test by statistical analysis because of the small frequencies As micropapillomatosis almost exclusively occurs in cases which also exhibit metaplasia (17) the same factor *may be responsible for both changes*

When estimating the occurrence of the different changes and particularly the extent of metaplasia in the different materials it has to be considered that all control specimens and the majority of the carcinoma specimens consisted of one complete lung whereas the bronchiectasis and tuberculosis specimens mostly were composed of lobectomy specimens (Table 1) This may mean that if complete lungs had been available also in the inflammatory groups and thus the sections could have been collected from a wider epithelial area the chance to detect a higher number of changes might have been improved It is however more probable that the present situation with all sections from the diseased part of the lung as it was the case in the inflammatory groups offered the highest yield of altered epithelium

√ SUMMARY

This investigation is a comparative histological and sputumcytological study of the occurrence of epithelial changes in the bronchi in a control series and in cases with 1) bronchogenic epidermoid or undifferentiated carcinoma 2) primary pulmonary adenocarcinoma 3) bronchiectasis and 4) active tuberculosis The epithelial alterations studied are the histological changes slit formation in columnar epithelium and low epithelium (result of expulsion of degenerated surface epithelium) and the connected cytological phenomenon ACCI (occurrence of an abnormally large number of columnar cells in cytological specimens with various degenerative alterations) Furthermore metaplasia with and without cellular atypia the histological alteration micropapillomatosis and also the extent of metaplasia has been studied

The results show that the squamous undifferentiated carcinoma group exhibited the highest frequency with respect to all changes whereas the bronchiectasis tuberculosis and adenocarcinoma groups took an intermediate position between these and the controls The great extent of metaplasia in the squamous undifferentiated carcinoma series emphasized still more the difference between this group and the other groups

The clear difference between the adenocarcinoma and the squamous undifferentiated carcinoma groups concerning all changes studied is in close conformity with the opinion that extrinsic factors affecting the surface epithelium possess much greater carcinogenic significance in the latter group

The chronic factors which injure the epithelium and seem to be active in chronic inflammatory lung disease cause an increased rate of epithelial metaplasia These factors may accordingly be of importance by creating a basis for carcinogenesis The higher incidence of metaplasia and particularly of epithelial atypia in squamous undifferentiated carcinoma indicates that in this instance additional causative elements have been added

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FIBRIN DEPOSITS ON THE SYNCYTIUM OF THE NORMAL HUMAN PLACENTA EVIDENCE OF THEIR THROMBOGENIC ORIGIN

By

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Received 16 viii 67

Deposition in the human placenta of a material considered to be fibrin was first described by *Langhans* in 1877 (57) He found a layer of this material on the chorion plate composed of two parts one compact component directly overlying the chorion and another fibrillary stratum bordering the maternal blood space Later *Ackermann* (1 2) observed that what appeared to be fibrin often covered parts of the surface of the villi *Eberhardt* (28) found fibrin deposits within the villi between the trophoblast and the stroma

There has been disagreement as to the origin of these fibrin or fibrin like deposits *Langhans* (57) claimed that the layer covering the chorion plate was fibrin derived from maternal blood As to the material associated with villi a similar view was adopted by *Eberhardt* (28) *Eden* (30) *Huguenin* (51) and *Grosser* (41) *Schickele* (87) considered the material between the villi to be fibrin derived from maternal blood whereas that within the villi was fibrin of foetal origin. *Kline* (56) claimed that the fibrin masses were of foetal origin extending from foetal vessels through breaks in the trophoblast secondarily the fibrin masses became covered by syncytium growing from intact villi or trunks However he rarely observed thrombi in foetal vessels

Siddall & Hartman (92) *Huber et al* (50) and *Fox* (33) suggested that the fibrin deposition was simply due to stasis of maternal blood in the intervillous space

Ashworth & Stouffer (8) observed a large number of blood platelets in association with recent deposits of fibrin and described the lesions as thrombi In older deposits platelets were no longer apparent They showed that the syncytium was often lacking at the site of attachment of the thrombi Ultrastructurally *Boyd & Hamilton* (16) f und fibrin (fibrinoid) on the surface of villi It appeared as a dense network and an occasional platelet could be identified in the interstices

McKay et al (62) related the deposition of fibrin to a focal loss of [illegible] activity of the syncytium assumed to be present normally Later *M* [illegible] *berg* (61) induced accumulation of platelet and fibrin in rat placent [illegible] in oxidized lipid and they claimed that th dep sits ould b [illegible] generation of the trophoblast

Another group of authors have quest [illegible] d whether th mat [illegible] ted on the chorion plate and [illegible] is fibrin Alterna [illegible] suggestio [illegible] that the deposits re cellular [illegible] product of the t [illegible] ast (40 [illegible] trophoblastic cell (9) or the result [illegible] immu [illegible] on (19)

The purpose of the present st[illegible] to evalu[illegible] th n ture of [illegible] deposits on the chorion plate an[illegible] in [illegible] human [illegible] acentas using bo[illegible]

light and electron microscopy Evidence will be presented that the deposits are thrombi formed from maternal blood and undergoing secondary changes

MATERIAL AND METHODS

Thirty three mature and 26 immature placentas were examined by light microscopy only The immature placentas were obtained by therapeutic abortions in women 8 to 24 weeks pregnant They had no somatic disorders and the pregnancy was interrupted on psychiatric indications Immediately after delivery the placentas were gently rinsed in isotonic saline and cut in slices Specimens were taken from the central and peripheral parts fixed in buffered formalin or Helly s solution for 24 hours dehydrated and embedded in paraffin The following stains were used

(1) Haematoxylin azo phloxine (HP)
(2) Lendrum s Martius scarlet blue method (MSB) (58)
(3) *Mallory s phosphotungstic acid haematoxylin method (PTAH)*
(4) Van Gieson s stain

Three mature and 3 immature placentas of 10 to 13 weeks were examined both by light and electron microscopy For electron microscopy tissue blocks from the basal middle and upper parts of the placenta were removed immediately after delivery They were fixed for 1½–2 hours in chilled isotonic 1¼ per cent glutaraldehyde in M/20 phosphate buffer (pH 7 4) and post fixed for 1¼ hour in 1 per cent isotonic osmic tetroxide (21) and embedded in Epon 812 One micron thick sections were cut on a Huxley ultramicrotome (Cambridge Inst Co) and stained with toluidine blue for orientation by light microscopy Suitable areas were selected and ultrathin *sections stained with uranyl acetate and lead citrate (81)* They were *examined in* a Zeiss EM-9 electron microscope

RESULTS

The findings in placentas at 8 weeks of pregnancy and later up to term were essentially the same although quantitative differences existed Unless otherwise stated the following description applies to all specimens studied

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Figs 1–4

Fig 1 Placenta at term Loosely packed platelet aggregates are apparently floating free in the intervillous space At lower left (arrow) a tightly packed platelet aggregate attached to the syncytium of a villus MSB × 600

Fig 2 Placenta at 17 weeks Small projecting platelet thrombus on the syncytium The thrombus is covered by a dark fibrin cap The syncytium is not definitely altered MSB × 500

Fig 3 Placenta at 8 weeks Flat deposit consisting of a basal fibrin layer and a *superficial* platelet aggregate The underlying trophoblast is thin the syncytial cytoplasm is vacuolated and the nuclei are pyknotic MSB × 500

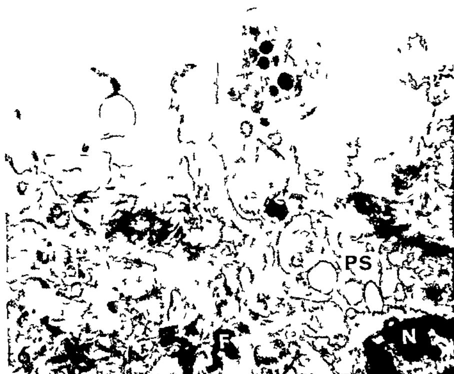
Fig 4 Placenta at term Small platelet aggregate attached to the syncytium (S) *One of the platelets (Pl T) has partly lost its organelles Between the platelets* and surface of the syncytium *there* is a flocculent granular to fibrillar substance of moderate electron density In *some* fibrillary tracts a cross striation with periodicity of about 230 Å can be discerned *(arrows)* The syncytium shows atrophy of microvilli and some dilatation *of endoplasmic* reticulum In insert a single platelet is in contact with the syncytium pseudopod (arrow) buried between the *microvilli* × 12 000 (insert × *9 000)*

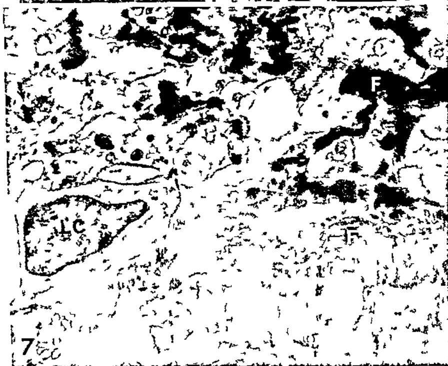

1


2


3


PLT
S
S


PS
N
F
6


F
LC
7

Recent Platelet Fibrin Thrombi

Light microscopy of immature and mature placentas revealed some apparently free floating platelet aggregates in the intervillous space (Fig 1) Other platelet aggregates were adhering to the syncytium of the chorion plate or villi (Fig 1) Commonly the platelet aggregates were surrounded and/or traversed by fibrin strands identified by characteristic staining properties in MSB and PTAH stains (Fig 2) Some deposits were flat and seemed to be composed entirely of fibrin or they might consist of two layers one basal layer of fibrin and another superficial one composed of platelets (Fig 3) Some neutrophil granulocytes and a few other leucocytes or erythrocytes adhered to the surface or were embedded within the mass

No thrombi were found in the foetal capillaries of the villi

Electron microscopy of the placentas revealed single or small groups of platelets in contact with the syncytium (Figs 4 and 19 insert) Even single leucocytes or red blood cells appeared occasionally to be attached to the syncytium The platelets or the other blood cells were either partly buried between projecting microvilli or they seemed to compress and bend the microvilli The distance between the superficial membranes of the platelets or cells and that of the syncytium varied considerably Usually in a shorter or longer part of the circumference of the platelets or cells the distance between adjacent membranes was only 2–300 Å *i e* about the same as between adhering cells in general Some of the platelets showed moderate morphological changes such as pseudopod formation and beginning loss of organelles On the surface of the syncytium at sites of adhering platelets and between loosely aggregated platelets there was occasionally a flocculent granular to fibrillar substance of moderate electron density (Fig 4) In areas where the fibrillary structure was distinct a cross striation with periodicity of about 230 Å was observed indicative of fibrin

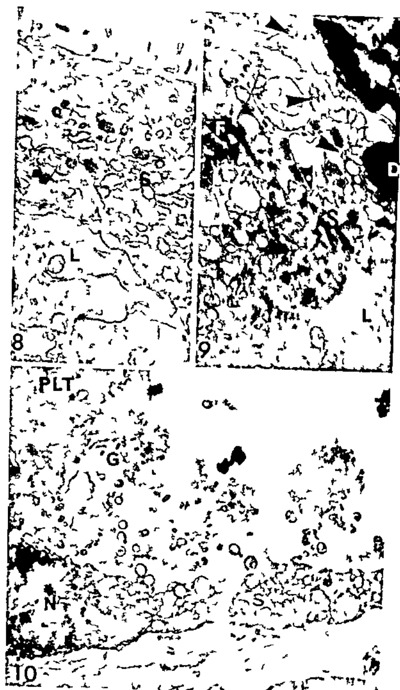
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Figs 8–10

Fig 8 Placenta at 13 weeks For comparison normal syncytium (S) and cytotrophoblast (L) × 12 000

Fig 9 Placenta at 13 weeks The deepest part of the deposit seen in Figs 6 and 7 To the left there is a fibrin net (F) with vacuoles and other cellular debris The syncytium (S) is nearly destroyed and poorly outlined Its outer border is probably along the arrows Within the cytoplasm there are vacuoles and fibrin strands some of which appear to be in direct continuity with the fibrin in overlying deposit A dense body (D) probably lipid is seen to the right Part of a syncytial nucleus at upper right (N) The cytotrophoblast (L) at the bottom is nearly unchanged × 12 000

Fig 10 Placenta at 11 weeks Fragmenting granular mass (C) encompasses membranes vacuoles and platelet (PLT) This deposit is probably undergoing spontaneous lysis The syncytium (S) shows shrinkage loss of microvilli and marked vacuolization Vacuoles appear to be released from the syncytium × 12 000


F
D
S
L
8
9
PLT
G
N
S
10

Electron microscopy of the large hyaline deposits showed that they were mostly composed of compact granular masses of moderate electron density (Fig 15) Cellular debris including myelin figures or more or less preserved platelets were intermingled (Fig 15) Occasionally strands of fibrin with characteristic cross striation could be discerned within the mass Towards the surface the granular mass merged with fibrin associated with platelets and cellular debris (Fig 16) In other instances the granular masses were covered by platelets (Fig 17)

Coagulation Thrombi

In a few instances masses of red blood cells trapped in fibrin nets were found in the intervillous space These structures had an appearance of coagulation thrombi Ususally they were connected at one or more points with a villus or a platelet fibrin thrombus covering a villus

Trophoblastic Changes

Light microscopy showed that the syncytium underlying the platelet fibrin thrombi was sometimes normal but usually it showed shrinkage altered stainability of the cytoplasm and pycnosis of the nuclei (Fig 3) In some areas there was no syncytium underneath the deposit instead the deposit was partly covered by proliferating syncytium The hyaline deposits were likewise partly or entirely covered by syncytium (Figs 11 and 14)

The cytotrophoblasts in association with the platelet fibrin thrombi were less altered but occasionally they showed signs of proliferation This was more evident in the hyaline deposits (Fig 11)

In the electron micrographs the syncytium underlying the small platelet aggregates was either without changes or more often it showed atrophy of the microvilli and dilatation of the endoplasmic reticulum (Fig 4) The syncytium showed no breaks and the basement membrane or collagen fibres of the chorion stroma were not denuded in association with these aggregates

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Figs 15–17

Fig 15 Placenta at term Granular deposit (G) of moderate electron density with a lacuna containing platelets (PLT) and a myelin figure (M) Note the close contact between the platelets and the myelin figure In insert fibrillary tracts are seen within a granular mass A cross striation of about 230 Å may be discerned (arrow) × 12 000 (insert × 36 000)

Fig 16 Placenta at term Near the surface of a granular deposit (G) it merges with fibrin strands (F) × 36 000

Fig 17 Placenta at term. The surface of a granular deposit (G) is covered by loosely aggregated platelets (PLT) × 12 000


M
PLT
PLT
15


F
G
16


PLT
G
1


F
S
S
18
S
S
PLT
S
G
G

The syncytium underneath the layered platelet fibrin thrombi showed various degrees of structural alterations from slight swelling of the endoplasmic reticulum and disappearance of microvilli up to shrinkage marked vacuolization increased electron density of the cytoplasm and blurring or loss of organelles (Figs 5 9 and 10) Intracellular dense bodies and myelin figures were also encountered The cell membrane could show multiple gaps and vesicles seemed to be released from the syncytium (Figs 9 and 10) In some areas there were even intracellular fibrin strands occasionally in direct continuity with fibrin strands of the overlying thrombus (Fig 9)

Similar changes in the syncytium could be found also in areas not covered by any thrombus (Fig 18)

The syncytium covering some of the deposits contained as usual many mitochondria but otherwise the cells contained relatively few organelles (Fig 19) The endoplasmic reticulum was dilated and the microvilli were few and short At times platelets were adhering to the outer surface of this syncytium as well (Fig 19 insert)

In the immature placentas the cytotrophoblasts were usually intact underneath the altered syncytium covered by platelet fibrin thrombi (Fig 5) Occasionally the cytotrophoblasts showed changes similar to those of the syncytium but to a mild degree The cytotrophoblastic cells within the hyaline deposits were well preserved

DISCUSSION

The deposits on the syncytium of the human placenta were composed of more or less altered platelets and fibrin Thus we have been able to confirm the observations by *Ashworth & Stouffer* (8) that the earliest deposits in placenta are thrombi They have the appearance of platelet thrombi in various stages of transformation to fibrin thrombi (34 52 55 91)

Bizzozero (12) and *Eberth & Schimmelbusch* (29) showed that the initial step in thrombosis is adherence of platelets to the vascular wall followed by aggregation of additional platelets That the same may happen in the placenta is evident from the observation of platelet

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Figs 18–19

Fig 18 Placenta at term Syncytium (S) th loss of mit l ι n l la to ether with appearance of vacuoles myelin fig re nd a f w d n e bodies The n cytium s not covered b any dep t } t what app rs to be strand of fibrin (F) are in contact with th rf ce h s m y be a remnant of a lysed deposit × 1 000

Fig 19 Placenta t term Syncytium (S) ov a granul r d posit (G) The syncytium is rich in mitochondria usual but o l the or an lles are rel tively few In insert a platel t Pl F is n t ι t with syncytium (S) overlying a g anular leposit (1 0 0 (in ι t 9 000)

aggregates apparently sticking to the syncytium The occurrence of fibrin in association even with some of the smallest aggregates cannot be taken as an indication that fibrin formation is the primary event in placentar thrombosis Single platelets or small platelet aggregates were seen to be in contact with the syncytium without the presence of fibrin In vessels observation of the earliest stages of thrombus formation has not shown fibrin in the region in which platelets were in contact with the vessel wall (36 75) On the other hand platelet aggregation induced by adenosine diphosphate (ADP) has been shown to accelerate clotting (20 65) and morphologically fibrin formation *in vivo* is closely associated with platelet aggregates (52)

The increasing amount of fibrin in association with the thrombi and the destruction of the platelets are probably not caused by any influence from the underlying tissue since *Hovig et al* (49) observed the same changes with time in thrombi resting on a silicone coated plastic surface in an extracorporeal shunt More likely the changes are related to coagulation of maternal plasma seeping into the mass Thrombin not only leads to fibrin formation but may also cause platelet destruction (47 103) As in experimental thrombi (49 55) the soaking of the mass with plasma appeared to be facilitated by separation of the platelets in its deeper parts Plasma must have penetrated even into the underlying syncytium judged by the occurrence of intracytoplasmic fibrin Accordingly *Shirasawa* (91) found fibrin within endothelial cells underneath platelet fibrin thrombi in vessels

The large placental deposits consisted mainly of a hyaline substance which by electron microscopy was shown to have a granular structure the electron density of which was less than that of fibrin fibrils Several observations suggest that this material is derived from fibrin or a closely related substance it stains like fibrin by Lendrum s MSB stain in part it also stains like fibrin by the PTAH method fluorescein conjugated rabbit anti human fibrin is bound both to fibrin strands and to the hyaline substance (63) finally by electron microscopy bundles of fibrils with a cross striation of about 230 Å were found within and on top of the granular masses often merging with it Masses ultrastructurally identical to those described here have been termed fibrinoid by *Farquhar et al* (31) *Vassalli et al* (102) and *Ooneda et al* (71) In the two last mentioned papers fibrinoid is considered to be mainly composed of or derived from fibrin The fact that the granular masses predominantly were found in large deposits in mature placentas may indicate that they represent aged fibrin The finding of cellular debris even of an occasional more or less preserved platelet within the large deposits indicate that these masses are orginally derived from platelet thrombi

The presence of thrombi or layers of thrombi of obviously different ages suggests that placental thrombosis is a continuous or repeatedly recurring process as it is in intravascular situations (52)

Three sets of factors connected with the maternal blood circulation through placenta will conceivably promote thrombus formation

1) Irregularities of the blood flow in the intervillous space
2) Increased coagulation activity of the maternal blood and
3) Decreased fibrinolytic activity

In each placental cotyledon the villi are arranged in the so called "drum system They are anchored to the periphery of the cotyledon leaving the central part relatively poor in terminal villi (105) Frequently the utero placental arteries open into the centre of the maternal cotyledons (13 95) *i e* in the middle of the drum system (107) Blood is ejected like a jet (14) under high pressure (100 mm Hg) (19) The blood jet reaches the chorion plate without notable lateral dispersion (14 107) In the subchorionic region the circulation is slowed (77) and from there the blood advances slowly through the intervillous space (14) It drains via the utero placental veins which originate randomly in the basal plate (18 42 76 97 106) At term the diastolic pressure in the intervillous space is about 10 mm Hg earlier in pregnancy it is about 5–6 mm Hg (4)

These circulatory conditions are bound to give marked irregularities of flow First the arterial blood jet which is forced into a blood mass with a slow general movement in the opposite direction will entail very high velocity gradients and eddy formation peripheral to the jet Second as the blood jet reaches the chorion plate the flow is split and the direction and velocity is abruptly changed Third the villi projecting into the maternal blood space are forming obstacles to the flow Behind these obstacles eddies are probably again formed The significance of such flow disturbances for thrombus formation has been repeatedly stressed (6 26 29 52 64) More specifically *Dintenfass & Rosenberg* (27) showed that high velocity gradients will favour platelet aggregation This may be related to the greater chance of collision between the formed elements of the blood in such a flow Not only will this bring the formed elements close together but they may be mechanically damaged Because of their large volume and their fragility (22) the red blood cells may be the most important elements in this connection Mechanically damaged erythrocytes release ADP (43) which cause the platelets to aggregate (37 44) These mechanisms may possibly tend to give free floating platelet aggregates Such aggregates were apparently encountered in the placenta Secondly platelet aggregates may adhere to the syncytium just as small mural platelet thrombi may arise secondary to free floating aggregates in the microcirculation (54) However the observed apparently free floating aggregates could also be detached fragments from growing platelet thrombi attached somewhere to placental structures

Anoth[illegible] which may intensify the tendenc[illegible] to thrombosis in a turbulen[illegible] such a flow seems to favour an indiscriminate

deposition of any flow born material on the wall (82) The formed elements are probably thrown against the lining cells and thereby perhaps damaging both themselves and the syncytium Such collision damage will presumably be marked in the areas where the arterial blood jet reaches the chorion plate In favour of the view that this mechanism is important is the observation that thrombotic deposits were particularly constant and extensive on the chorion plate Further single red and white *blood cells as well as single platelets were observed to be in contact* with the syncytium indicating that this attachment is a rather unspecific phenomenon perhaps dependent on mechanical factors inherent in the flow pattern Due to the particular tendency of platelets to aggregate it is possible that one or a few single adhering platelets may become the nidus for a thrombotic process

Whether the possible damage to the syncytium plays any role for the initiation of the thrombosis is difficult to evaluate Single platelets or small platelet aggregates were occasionally attached to morphologically unaltered syncytium Underneath most of the deposits were signs of injury to the syncytium revealed by shrinkage marked vacuolization atrophy of microvilli and loss of organelles Occasionally the syncytium was lacking However these changes could well be secondary to the thrombi caused by anoxia and/or by influence of factors released from the platelets (54 66) Altered but naked syncytium were sometimes encountered but this could be explained if we assume that an overlying thrombus had been dissolved The observation of fragmented deposits loosely connected with altered syncytium supports such a view

Immunological reactions at the border of the maternal blood and the foetal chorion tissue are probably not important for the placental thrombosis since trophoblastic cells do not express antigenicity (93)

Whatever the mechanism of initiation of the thrombotic process primary aggregation or primary adhesion the platelets appeared to stick to an uninterrupted syncytium with a continuous layer of cytotrophoblasts underneath There was no possibility of contact between the platelets and the basement membrane or the collagen fibres of the chorion stroma In vessels adherence to these connective tissue components are considered to be important for the initiation of thrombosis (7 15 35 36 94) However at sites of disturbed flow in extracorporeal *shunts platelet masses may adhere to siliconized plastic surfaces (64)* or to fibrin (48) Furthermore when the surface of coagulation thrombi are exposed to flowing blood platelet aggregates may stick to the thrombus (5 82 100) In the placenta platelets seemed to adhere not only to normal or altered syncytium but also to the presumably aged fibrin All these observations give strong evidence for the view that platelet adherence *in vivo* may largely be independent of the character of the exposed surface or material and is rather governed by the flow pattern

The second set of factors which probably contributes to the pre

disposition to thrombosis in the human placenta is associated with the increased coagulation tendency assumed to be present during pregnancy in general and in placenta in particular The fibrinogen concentration is increased from the first trimester (17 25 39 74 83) In late pregnancy slightly elevated values of prothrombin (68 79 101) and Factor V (101) are seen together with a marked increase in Factor VII (24 68 72 101) a moderate increase in Factor VIII (69 99 101) an increase in Factor IX (69 78 101) and Factor X (69 72) *Russ et al* (84) examined women in late pregnancy and found an increase in plasma phospholipids *Renkonen* (80) and *Nelson* (67) obtained the same results when they determined the serum phospholipids These coagulation studies have not been performed early in pregnancy when the thrombotic tendency appear to be already manifest

There is no significant change in platelet counts during pregnancy (25 68 79 96 99 101) *McKay et al* (60) found platelet adhesiveness slightly elevated but the results were not statistically significant

The content of tissue thromboplastin (88) is very high locally in the placenta and decidua The thromboplastic activity in chorion cells is associated with an intracellular particulate material capable of accelerating clotting in high dilutions (23)

If the high values of coagulation factors generally and locally reflect a more easily triggered or faster coagulation this may favour the thrombotic process both by a more rapid formation and stabilization of platelet aggregates (53) and by hastening the fibrin precipitation in association with these aggregates In spite of the assumed increased coagulation activity coagulation thrombi in the intervillous space were not frequent The ones observed were often continuous with platelet fibrin thrombi *i e*, they were coagulation parts of mixed thrombi However we cannot exclude that an occasional coagulation thrombus may have formed independent of platelet aggregation in areas of retarded flow (52)

The third set of factors which may favour the occurrence of thrombi in the human placenta is connected with the marked reduction of fibrinolytic activity during the pregnancy (17 39 59 89 90 108) *Albrechtsen* (3) and *Beller et al* (11) found that tissue activators of plasminogen were absent in human placenta and decidua However *Phillips et al* (73) found some activator activity in human placental extracts

In the placentas studied here there was morphological evidence of some degree of thrombolysis (4 52 85 86) but to a large extent the masses appeared to remain undissolved That may indicate that the spontaneous fibrinolytic activity in placenta is relatively weak Lysis by neutrophil granulocytes does not seem to be of importance either since they were not attracted by the thrombi in large numbers

If we accept that the deposits are thrombi promoted by circumstances inherent in certain characteristics of the human placenta and the preg

nant state why do the deposits not become organized as thrombi in vessels? Thus there was no invasion of fibroblasts or smooth muscle cells even though animal studies on the organization of thrombi on synthetic material exposed to blood flow have given evidence that mesenchymal cells in organizing thrombi may at least partly be derived from blood cells (38 55 70 98) It is possible that the explanation of this is related to the fact that the thrombi seemed to attract relatively few monocytes or macrophages On the other hand the proliferation of cytotrophoblastic cells also described by previous authors (32 62 87 104) may be considered an equivalent to organization in this special organ

Syncytium formed partly or entirely the outer lining of many deposits as shown by *Kline* (56) *Ashworth & Stouffer* (8) and *Zacks & Blazar* (109) Theoretically this could be regarded as a precipitation of fibrin underneath the syncytium secondary to an increase of its permeability or as a result of intravillous fibrin deposit due to other mechanisms such as thrombosis of foetal capillaries (56 87) The present study did not provide any evidence in support of these views Even the villus like apparently isolated deposits partly or entirely covered by syncytium need not be explained by intravillous fibrin deposition because serial sectioning revealed that they were cross sections of polyp like thrombi attached to a villus or to the chorion plate The syncytialization of the deposits must be considered an active proliferation analogous to the endothelial overgrowth of thrombi in vessels

SUMMARY

Studies by light and electron microscopy show that the deposits on the syncytium of villi and chorion plate in the normal human placenta are thrombi derived from maternal blood They begin as platelet thrombi and secondarily fibrin is formed

The thrombosis is *not* initiated by an interaction between platelets and exposed basement membrane or collagen fibres since the platelets adhere to an uninterrupted syncytium with no possibility of contact with the underlying connective tissue components

In more advanced stages the deposits are masses without characteristic structure They are considered to represent aged fibrin The following observations support this view the masses were predominantly found in large deposits and in mature placentas histologically they were at least partly stained as fibrin they stained positively with fluorescein conjugated rabbit anti human fibrin (unpublished observations) and electron microscopy showed that the masses merged with fibrin strands The occasional finding of more or less preserved platelets within the mass indicate that they are originally derived from platelet thrombi

The usual organization of thrombi does not take place However

cytotrophoblastic cells proliferate into the deposits and syncytium is partly or entirely forming the outer lining analogous to endothelialization of thrombi in vessels

The tendency to thrombosis in placenta is probably caused by an extraordinary flow pattern of the maternal blood increased concentration of maternal coagulation factors and decreased maternal fibrinolytic activity

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ential counts were made on smears from the peritoneal fluid from the following control groups

(3) normal mice
(4) normal mice with intraperitoneal injection of saline
(5) previously immunized mice without further treatment
(6) previously non specifically stimulated mice
(7) previously non specifically stimulated mice with intraperitoneal tumour transplants

Group (7) was added to exclude the possibility that a special contact phenomenon (vide infra) observed in the immunized mice with intraperitoneal tumour transplants was due to a general activation of phagocytic activity by the previous tail tumour In this control group the phagocytic activity was stimulated with Glycerol trioleate (Triolein) prior to tumour injection A Triolein suspension was prepared as stated by *Stuart et al* (1960) and 0 05 ml (9 2 mg of Triolein) was given intravenously The effect on the reticulo endothelial system was measured 24 hours later by means of the phagocytic index according to the method described by *Bio i et al* (1953) Each mouse received 0 1 ml of the carbon suspension in saline (8 mg/ml) intravenously The mean phagocytic index in 4 stimulated mice was 0 098 ± 0 027 whereas in 4 untreated controls it was 0 039 ± 0 006 This difference was significant (t = 4 110 P < 0 05) Twenty four hours after a similar dose of Triolein ?0 mice received EAC intraperitoneally The peritoneal fluid of these mice was examined after ½ ? 6 ?4 and 48 hours as stated above.

Splenic weights and haemoglobin content The spleen was removed in tumour bearing mice and in the controls and the relative splenic weight calculated as previously described (*Thunold* 1967 b) It has been found that the pleen of tumour bearing mice contains a large number of red cells (*Thunold* 1967a) As the effect of this high red cell content on the splenic enlargement was not clear haemoglobin estimation was made on splenic tissue from the tumour bearing mice in order to get an impression of the amount of red cells present The procedure was as follows a piece of splenic tissue was weighed ground in a mortar and su pended in 4 ml 0 05 per cent ammonia The suspension was left for 24 hours at 4 C and then centrifuged The amount of haemoglobin in the supernatant was determined in a Unicam Spectrophotometer at 540 mμ From this value the haemoglobin content in the spleen was calculated (mg Hgb/100 mg splenic tissue)

RESULTS

Cytology

Rosettes of lymphocytes were seen to surround well preserved tumour cells (*Fig 1 a b*) as well as injured tumour cells (*Fig 1 c*) between ½ hour and 5 days after tumour injection but rarely later *Table 1* shows the number of lymphocytes rosettes in ten consecutive high power fields and it appears that the phenomenon was more frequent in immunized than in control mice and that it appeared later in the controls

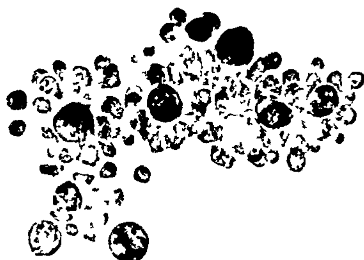
Rosettes of histiocytes and tumour c lls (*Fig 2*) occured occasionally from the second day in both immun z d and contr l mice However this phenomenon showed no diff rence between the two groups

Previous stimulation with Triolein did not give c anges different from thos n in the previously untreated controls hagocytosis of tumour ce is not seen in any of the groups

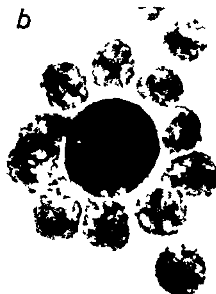
Differenti rls

Table ? sh w he mean and the range of the different cell types in the periton t t from normal mice The individual variation in the

a



b



c



Fig 1

Cells from an immunized mouse ½ hour after injection of Ehrlich's ascites carcinoma May Grunwald Giemsa stain

a Aggregate of lymphocytes and morphologically well preserved tumour cells × 610

b Greater magnification (× 1500) of lymphocytes surrounding well preserved tumour cell

c Lymphocytes surrounding injured tumor cell. × 1500

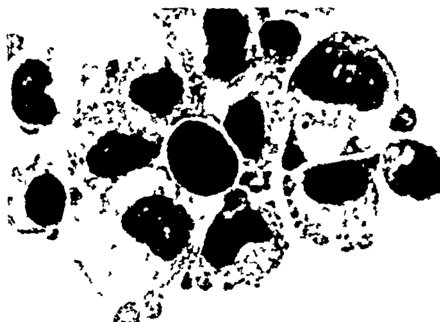


Fig 2

Cells from an immunized mouse 2 days after injection of Ehrlich s ascites carcinoma Tumour cell in close contact with histiocytes There is no evidence of phagocytosis. May Grunwald Giemsa × 1 00

TABLE 1

The Number of Rosettes between Tumour Cells and Lymphocytes in Smears from the Peritoneal Fluid in Immuni ed and Control Mice with Ehrlich s Ascites Carcinoma

Groups of mice	Time after tumour injection Hours ½	2	6	24	48	Days 5
Immunized	12 ± 4 0	14 0 ± 6 5	9 0 ± 4 5	6 8 ± 2 0	3 8 ± 3 5	3 8 ± 1 5
Control	2 0 ± 1 0	3 3 ± 2 0	2 5 ± 0 5	0 8 ± 0 5	1 8 ± 1 5	1 8 ± 1 0

Ten consecutive high power fields (× 800) were counted in each smear
Entries means of the individual groups ± SD

TA [illegible] [illegible]

The Ranges (p [illegible] ent) and Me ns for th D ff rent Cell Ty [illegible] s of the Peritoneal Flui [illegible] [illegible] No [illegible] al Mi e

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number of lymphocytes and histiocytes was great Injection of saline alone to normal mice caused a slight rise in the number of neutrophils with return to normal values in 2–3 days The peritoneal fluid from mice in which a primary tail tumour had been removed and mice stimulated with Triolein showed a similar cell distribution to that in the normal mice

In general the relative distribution of tumour cells and host cells was similar in immunized and control mice after intraperitoneal injection of EAC and it was in agreement with findings in previously untreated mice reported by *Klein* (1951) As the absolute amount of tumour cells and host cells was different in immunized and control mice from the tenth day (*Thunold*, 1967 (c)) comparison of relative values was of only limited value in late transplants

However some difference between the groups was seen in the early phases of tumour growth The percentage of *well preserved tumour cells* was lower in immunized mice than in the controls The difference was only significant on the second day (imm 11 7 ± 5 6 per cent contr 37 3 ± 9 1 per cent $t = 4\,803$ $P < 0\,01$) Contrary to this the relative number of *injured tumour cells* was larger in immunized than in control mice at ½ hour (imm 3 4 ± 1 5 per cent contr 1 2 ± 0 5 per cent $t = 2\,550$ $P < 0\,05$) These cells, similar to those previously described (*Thunold* 1966a fig 3b) were large with marked cytoplasmatic damage while the nucleus and the nucleoli were relatively more intact

In addition to this *eosinophils* and *neutrophils* which were only occasionally present in normal peritoneal fluid appeared regularly between ½ hour and 5 days both in the controls (eos 0 4–1 5 per cent neutr 0 5–2 5 per cent) and in the immunized mice (eos 1 5–3 5 per cent neutr 0 5–1 7 per cent) There was no significant difference between the groups

The relative distribution of tumour cells and host cells in mice receiving EAC after earlier stimulation with Triolein was not different from that seen in previously untreated controls

Splenic Weights and Haemoglobin Content

Fig 3 shows the changes in relative splenic weight and splenic tissue haemoglobin after transplantation of EAC to control and immunized mice The standard deviation of the mean is only given when a significant deviation from normal values occured In the controls the splenic weight increased reaching its maximum after 10 days Subsequently it showed a tendency to decrease The haemoglobin content of the splenic tissue also increased but this reached its greatest value after 5 days At 10 days when the relative splenic weight was at its maximum the tissue haemoglobin had returned to normal values

In previously immunized mice the splenic weights were above nor

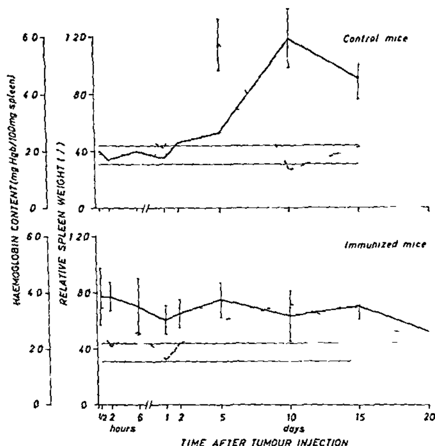


Fig 3

The mean relative splenic weight (——) and mean haemoglobin content (----) at various times after injection of Ehrlich's ascites carcinoma to untreated control and immunized mice The standard deviation is only given if a significant change from normal values is present The latter are given by the horizontally drawn and dotted lines which represent the limits of 2 standard deviations

mal and following intraperitoneal tumour transplantation no further increase could be seen The haemoglobin content of these spleens did not change from normal values after tumour injection

The changes in splenic weight and haemoglobin content in mice treated with Triolein prior to tumour in ection were not different from those in the previously untreated c nt l There was no increase in relative splenic weight after intr p riten i n of saline only

DISCUSSION

Transplantation of EAC into the ... followed by e c sion of the tumour has been shown to inhibit a ... quent ... moreal r t of the

same tumour (*Thunold*, 1967 (c)) In the present experiment the intraperitoneal cellular reaction and changes in splenic weight following the growth of this homotransplantable tumour have been examined The changes observed have been compared with those reported in *in vivo* and *in vitro* studies on homografts of known antigenicity

There was a marked aggregation of lymphocytes to tumour cells as early as ½ hour after transplantation and during the first 5 days of tumour growth in immunized mice Similar rosettes of lymphocytes and tumour cells were found in the previously untreated mice after tumour transplantation but this response was significantly less marked than that observed in the immunized group Lymphocytes are not known to possess phagocytic properties Neither did stimulation with Triolein prior to tumour transplantation give any increase in the number of such rosettes It is therefore unlikely that they should be due to a general activation of the phagocytic capacity by the tail tumour Thus the strong aggregation of lymphoid cells to the tumour cells in the immunized mice may probably be attributed to a sensitizing effect of the previous tail graft This is also suggested by the immediate recognition of the tumour cells which is in agreement with the behaviour of primed lymphocytes in the immune response to a specific antigen These findings show that EAC initiates cellular reactions in the hosts similar to those seen in ordinary homografts and that these reactions can be significantly strengthened by previous treatment of the mice with the same tumour EAC grows non specifically and probably only possesses weak antigenic strength These findings however show that there is a residual antigenicity which can be visualized after pre immunization

The weak lymphocytic response found in the control mice has apparently none or only limited influence on the tumour growth On the other hand the simultaneous findings of tumour growth inhibition and rosette phenomena in the immunized mice points to a cell bound inhibiting effect on the tumour The morphological study also indicates that a process involving surface contact between tumour cells and immune lymphoid cells is a main point in the immunized mice and may be of importance to the general tumour inhibition A cytotoxic effect of cellular factors may also be involved in the increase in number of injured tumour cells found ½ hour after transplantation in these mice However experiments have shown that immune lymphoid cells and tumour cells require 18–40 hours of incubation before cytotoxic effects occur (*Rosenau & Moon* 1961) Even if these *in vitro* results can not be directly compared to those obtained in the present experiment humoral antibodies and/or differences in complement level may thus be of greater importance in these *early* cytolytic reactions Such antibodies can also be involved in the outcome of tumour growth which would complicate the significance of the morphological results However it has been repeatedly shown in *in vitro* experiments that ascitic

sarcomas and carcinomas have a low degree of sensitivity to cytotoxic antibodies (reviewed by *Winn* 1962) and the only known *in vivo* effect of humoral antibodies in similar tumour systems is that of facilitating tumour growth (*Kaliss* 1958)

Thus even if humoral antibodies are implied the more likely mechanism in this experiment is an inhibiting effect of cell bound immunity A similar close contact between tumour cells and lymphocytes can be seen at the time of rejection of primary solid and ascitic homografts (*Kidd & Toolan* 1950 *Weaver et al* 1955 *Weaver* 1958) but the importance of an intimate surface contact between these cell types has been more clearly shown in tissue culture (*Rosenau & Moon* 1961) On the whole the present findings stress the role of the lymphoid cells in the immune response This is well established and recently reviewed by *Gowans & McGregor* (1965)

A weak rosette formation of tumour cells and histiocytes occured from the second day and with similar frequency in immunized mice control mice and in those treated with Triolein prior to tumour transplantation *Thus it seems that these cells play a part in the primary* reaction against the tumour As the reaction could not be strengthened by previous immunization of the mice it is most probable that the action of these cells is non specific and not immunologically determined A more pronounced aggregation of histiocytes to tumour cells than that obtained in the present experiment can be seen at the time of rejection of incompatible ascitic tumours transplanted subcutaneously or intraperitoneally and phagocytosis by histiocytes does not play a major part in the destruction of the tumours (*Gorer* 1956 *Amos* 1962 *Baker et al* 1962) even though it is accepted that histiocytes possess phagocytic properties (*Fagreus* 1960) The mode of action of the histiocytes in tumour homografting thus remains vague Recent experiments on the initiation of the immune response have shown however that antigen may be acted upon by histiocytes and that these cells play a part as carriers of antigenic stimulation to the lymphoid system (*Fishman* 1961)

Eosinophilic cells also appeared regularly in the early phase of tumour growth both in immunized *and in control mice* but they *were* not seen in close contact with the tumour cells Nevertheless this finding is pertinent to the question of tumour antigenicity in the present system Eosinophilic cell infiltration has been found in the homograft rejection reaction (*Rogers et al* 1953) and the more recent studies by *Litt* (1961 1962) provide convincing evidence that eosinophils are attracted by antigen antibody complexes According to this the present findings indicate that EAC is recognized as foreign and that antibodies are produced against it In contrast to the eosinophilic response early appearance of neutrophils could also be seen in saline treated controls *and may thus be due to a non specific irritant effect of the tumour cells*

During primary intraperitoneal growth of LAC an increase in splenic weight takes place which reaches its maximum after 10 days. It has previously been reported that such hyperplasia is associated with microscopical changes characteristic of an immunological stimulation (*Thunold* 1967 a, b). The increase in splenic weight, however, can only be accepted as a direct expression of immune response if the weight increase is due to a *proliferation of the lymphoid system*. An increase in phagocytic activity after tumour transplantation has been reported by *Old et al.* (1960) and *Thunold* (1967 a) discussed the significance of an increase in haematopoietic activity and blood content on the splenic weight increase. As far as the haemoglobin values can be taken as an expression of the red blood cell content, the present experiment shows that this may play a part in the splenomegaly in the early phase of tumour growth. It should be stressed that the haemoglobin content has returned to normal when the splenic weight is highest. This means that the main increase in splenic weight is due to proliferation of cell elements and not to the content of red cells. This weight increase also coincides with the increase in immunological function. The spleen is generally accepted as a major organ in the production of humoral antibodies, and the timing of the increase in weight in the present experiment is in agreement with the appearance of haemagglutinating antibodies after transplantation of other homografts (*Mitchison* 1955).

Splenomegaly was present in the immunized mice at the time of intraperitoneal tumour transplantation as an expression of an activated lymphoid system. The splenic weight remained above normal values during tumour growth. *In these mice tumour growth caused no further* increase in splenic weight or any increase in haemoglobin values. This was in contrast to the conditions in mice with a primary intraperitoneal tumour. The change in splenic weight in control and immunized mice confirm our findings of a local cellular primary and secondary immune response to the intraperitoneal tumour. It is also strikingly similar to features observed by *Andreini et al.* (1955) after primary and secondary transplantation of sarcoma I to incompatible hosts.

Histological evidence of immune reactions against LAC have been demonstrated in the spleen and lymph nodes of mice (*Thunold* 1967 a, b). The present study reveals a weak local cellular response to the tumour. These reactions have evidently no inhibiting effect on primary tumour growth. However, this experiment also shows that pre-immunization of the mice causes a marked lymphocytic reaction to a subsequent tumour graft. This is similar to the reactions found to occur when a primary homograft of known antigenicity is rejected and to the reaction between immune lymphoid cells and neoplastic target cells *in vitro*. The reaction is accompanied by increased resistance to the tumour.

SUMMARY

The cellular reactions and changes in splenic weight were studied after intraperitoneal injection of Ehrlich s ascites carcinoma in normal mice and in mice immunized with a tail transplant of the same tumour

The growth of EAC in previously untreated mice caused a weak reaction of lymphocytes histiocytes and eosinophils indicating a primary immune response The progressive growth of the tumour was not inhibited by these reactions In contrast to these findings a distinct aggregation of lymphocytes to the tumour cells was found in the immunized mice indicating a reaction between sensitized lymphocytes and tumour cells Simultaneous growth inhibition of the tumour was observed in this group This was probably due to a cytotoxic effect of cellular factors

Intraperitoneal tumour transplantation to normal mice gave a pronounced increase in splenic weight while an already existing splenomegaly in the immunized mice did not change significantly after intraperitoneal tumour injection The change in splenic weight is in agreement with the local cellular findings in control and pre immunized mice

The data of the present experiment indicate that the growth of EAC can be inhibited by local and general reactions which are similar to those shown in *in vivo* and *in vitro* studies on homografts of known antigenicity

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STUDIES ON THE PANCREATIC A_1 CELLS AND THEIR REACTIONS TO GASTRIN ADMINISTRATION

By

BIRGER PETERSSON

Received 29 viii 67

As in many other species the existence of A_1 A and B cells has been demonstrated also in the pancreatic islets of guinea pigs (*Petersson et al* 1962) For the proper identification of the different cell types it is necessary to re stain silver impregnated sections by a granule staining method (*Hellerström & Hellman* 1960) This re staining procedure has so far been used only on paraffin embedded pancreatic material In order to provide a more detailed morphological evaluation of the different islet cell types Epon embedding should be preferred to paraffin embedding shrinkage artifacts to develop after the former procedure being fewer than those to develop after the latter, besides Epon embedding permits the cutting of thinner sections Epon embedded pancreas has previously been used for the demonstration of A and B cells without removing the embedding medium (*Munger* 1961) This method is not suited for silver impregnation (*Petersson* 1966) By a recently described method it is however possible to remove the Epon before the staining procedure (*Lane & Europa* 1965) In the present work this method was applied to sections of guinea pig pancreas subsequently silver impregnated and re stained to demonstrate A_1 A_2 and B cells

While the function of the A_1 cells is obscure there is now strong evidence that the A_2 cells produce glucagon After long term administration of glucagon to guinea pigs there was a regressive change of the A_2 cell fraction while the A_1 cell fraction remained quite unchanged (*Petersson & Hellman* 1963) This view is also supported by data on the glucagon content of pure samples of A cells (*Unger et al* 1967) Since it has been suggested that the A cells may be related to the gastrin production (*Cavallero & Solcia* 1965 *Thiery & Bader* 1966) it seemed reasonable to test their reaction to gastrin administration In the present work the functional state of the islet cells was tested by caryometry after 5 days administration of gastrin to guinea pigs

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MATERIAL AND METHODS

Six male guinea pigs weighing 400–550 g were used in studies of the secretion granules in the different types of islet cells Pancreatic specimens from the animals were dis sected out and fixed in Zenker s solution (with and without glacial acetic acid) or Bouin s solution The pieces were then dehydrated and embedded in paraffin or Epon (*Luft* 1961) The paraffin embedded material was cut into 4 μ thick sections the Epon embedded material being cut with an ultramicrotome into 0 5–1 μ thick sections The paraffin was removed with xylol and the Epon with alcoholic NaOH solution as described by *Lane & Europa* (1965) The sections were first silver impregnated (*Hellerstrom & Hellman* 1960) and photographed for the subsequent identification of different cell types after re staining This was performed either with chrome haematoxylin and ponceau fuchsin (*Bencosme* 1952) aldehyde fuchsin trichrome (*Iazarus & Volk* 1962) phosphotungstic acid haematoxylin PTAH (*Lillie* 1954) or Heidenhain Azan staining (*Ferner* 1952) Consecutive sections were also directly granule stained using the same procedure

Every third hour throughout 5 days nine male guinea pigs received subcutaneous injections of gastrin (Leo Pharmaceutical Products Ballerup Denmark 135 Leo units/2 ml of physiological saline pH 6 9) according to the following schedule

Injection Nos	1–24	50 μl
Injection Nos	25–38	75 μl
Injection Nos	39–41	100 μl

Nine animals were used as controls and received a corresponding amount of physiological saline The animals were decapitated 1–2 hours after the last injection Two pieces of the pancreas one from the caput the other from the cauda were fixed in Bouin s solution dehydrated and embedded in paraffin in a uniform way (*Hellman* 1959) The pieces were cut into 7 μ thick sections

The gastrin effect on the islet cells was evaluated by caryometry The islets in the silver impregnated pancreatic sections were photographed the nuclear measure ments being performed after subsequent re staining with chrome haematoxylin and ponceau fuchsin (*Bencosme* 1952) In each animal the areas of the largest optical cross sections of 20 A_1 20 A_2 and 20 B cells were calculated after measuring the largest nuclear diameter and the diameter at right angles to the latter by means of an ocular screw micrometer at a magnification of 1875 X (cf *Hellman & Hellerstrom* 1959) The data were sampled from at least two islets in the caput and the same number in the cauda Only nuclei with their centres within the sections were measured

RESULTS

Thin sections of the Zenker fixed and Epon embedded pancreatic pieces were well suited for the distinct differentiation of A_1 A_2 and B cells by the methods used The cytoplasm of the A_1 cells was distinctly blackened after silver impregnation while the nuclei showed a weaker staining At high magnification the cytoplasm of these cells seemed to be filled with black grains After re staining with aldehyde fuchsin trichrome the A_1 cells displayed a bluish homogeneous colour but lacked the large blue granules characteristic of the B cells In the B cells the numbers of the blue granules varied greatly from only a few up to large numbers filling up the whole cytoplasm The A_2 cells were more difficult to identify when this staining method was applied to Epon embedded sections After re staining with chrome haematoxylin and ponceau fuchsin the A_1 cells had about the same appearance as the B cells even though the granulation was of a finer character (Figs 1 and 2) By contrast the A_2 cells had a distinct red cytoplasmic



Figs 1–4

Fig 1 An islet containing an A_1-cell with elongated cytoplasm Silver impregnation after Zenker fixation and paraffin embedding × 1450

Fig 2 The same islet after re-staining with chrome haematoxylin and ponceau fuchsin Note the finer granulation in the A_1 cell as compared with other islet cells × 1450

Fig 3 Part of an islet with three A_1 cells in the centre Silver impregnation after Bouin fixation and paraffin embedding × 1450

Fig 4 The same islet section as in Fig 3 after re-staining with *chrome* haematoxylin and ponceau fuchsin Note the pale appearance of the cytoplasm of the A_1 cells × 1450

staining After staining with PTAH or Heidenhain *Azan* it was not possible to distinguish A cells from B cells

In pancreatic sections from Zenker fixed and paraffin embedded material the A cells had a blue or green tint after staining with

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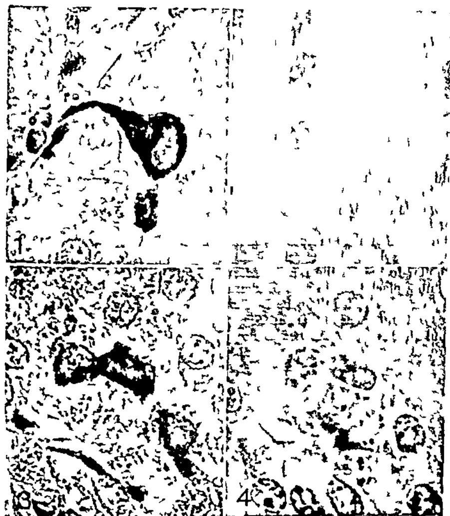


Fig 1–4

Fig 1 An islet containing an A_1-cell with elongated cytoplasm. Silver impregnation after Zenker fixation and paraffin embedding × 1450

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staining After staining with PTAH or Heidenhain Azan it was not possible to distinguish A_1 cells from B cells

In pancreatic sections from Zenker fixed and paraffin-embedded material the A_1 cells had a blue or green tint after staining with

TABLE 1

The Nuclear Areas of the A_1, A_2 and B Cells in Gastrin Injected and Control Groups The Mean Values ± Standard Errors Are Given in Arbitrary Units

Cell type	Nuclear area	
	Gastrin treated animals	*Control animals*
A_1 cells	1.23 ± 0.02	1.26 ± 0.04
A_2 cells	1.42 ± 0.03	1.48 ± 0.03
B cells	1.24 ± 0.02	1.25 ± 0.02

aldehyde fuchsin trichrome and ponceau fuchsin while the A_2 cells had a distinct red cytoplasm In addition to the A_1, A_2 and B cells a few cells were of a light appearance and they lacked the characteristics of the other cell types In the largest islets the number of these cells did not amount to more than one or two After staining with chrome haematoxylin and ponceau fuchsin the A_1 cells showed a bluish colour similar to that of the B cells After fixation in Zenker's solution containing glacial acetic acid or Bouin's solution the A_1 cells were of a lighter appearance than the B cells when stained with chrome haematoxylin and ponceau fuchsin (Figs 3 and 4) After staining with PTAH and Heidenhain Azan it was not possible to distinguish the A_1 cells from the B cells independently of the fixation used

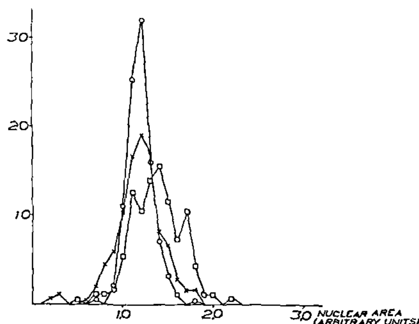


Fig 5

The frequency curve of the nuclear surface in arbitrary units of the A_1 (×—×) A_2 (□—□) and B (○—○) cells in the nine gastrin injected guinea pigs 20 observations from each animal

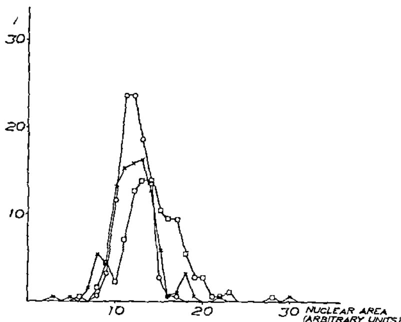


Fig 6

The frequency curve of the nuclear surface in arbitrary units of the A_1 (X—X) A_2 (□—□) and B (O—O) cells in the nine control animals 20 observations from each animal

At the start of the gastrin injections the experimental animals had a body weight of 517 ± 18 g the body weight of the controls bein, ɔ17 ± 17 g (mean values ± standard errors of the means) After 5 days of injections the respective body weights were 534 ± 16 g and 519 ± 22 g As shown in Table 1 the mean nuclear size of the A_1 cells in the gastrin injected group was 1 23 ± 0 02 and in the control group 1 26 ± 0 04 (arbitrary units) The values were not significantly different ($t = 0\ 8$ $P > 0\ 05$) The corresponding values for the A_2 cells were 1 42 ± 0 03 and 1 48 ± 0 03 ($t = 1\ 0$ $P > 0\ 05$) and for the B-cells 1 24 ± 0 02 and 1 25 ± 0 02 The frequency curves of the A_1 and A cell nuclear areas appeared similar in both animal groups and with a dislocation to the right of the A curves In both animal groups the B cell curves had a steeper rise and fall than the A cell curves (Figs 5 and 6)

DISCUSSION

It emerged from the present work that even for studies of the pancreatic islets in the light microscope plastic embedded material is preferable to paraffin embedded material owing to the negligible shrinkage artifacts and the possibilities of making thinner sections Aldehyde fuchsin and trichrome staining has previously been used on plastic-embedded pancreatic sections for the identification of the A

and B cells in the islets of Langerhans (*Munger* 1964) It is doubtful whether *Lee* (1967) was able to recognize A_1 cells with his silver impregnation method since he had a pH of 9 0 in the silver solution and the composition of the staining solution differed from that in the modified Davenport method In the present study A_1 A and B cells could be properly identified by the modified Davenport silver impregnation method and subsequent re staining with chrome haematoxylin and ponceau fuchsin (*Hellerstrom & Hellman* 1960) Although all these cell types can be satisfactorily demonstrated also after paraffin embedding the cells have a more distinct appearance after Epon embedding Besides the three cell types mentioned very few cells were characterized by a lack of cytoplasmic granules It is not reasonable at present to resignate these cells as a special cell type They many represent temporarily degranulated cells or degranulated parts of cells It also remains to be decided whether they have any connection with the few agranular cells observed under the electron microscope in the guinea pig islets (*Bencosme & Pease* 1958 *Caramia et al* 1965)

In his classical description of the guinea pig pancreas *Bensley* (1911) distinguished besides A and B cells also a third cell type which he called clear cells These cells lacked the granules characteristic of A or B cells It is reasonable to suppose that his A cells are identical with the A cells and it has been suggested that the clear cells are identical with the A_1 cells (*Petersson et al* 1962) The pale appearance of the A_1 cells often seen after fixation in Bouin s solution and subsequent staining with chrome haematoxylin and ponceau fuchsin may be explained by the presence of glacial acetic acid in the fixation solution (cf *Lazarus & Voll* 1962) This view is supported by the present finding that the A_1 cells often were of clear appearance even when Zenker s solution was complemented with glacial acetic acid The fixation solution used by *Bensley* (1911) also contained this acid After fixation in Bensley s solution it was not possible to apply successfully the silver impregnation method (*Petersson* 1966) D cells originally described in man (*Bloom* 1931) have been found only in low frequency in the islets of guinea pigs (*Thomas* 1937 *Gomori* 1939 *Ferner* 1952) Whether this cell type is identical with the A_1 cells also in the guinea pigs as has been claimed to be the case in certain other species (*Epple* 1963 *Fujita* 1964 *Solcia & Sampietro* 1965) remains to be settled In various other species a lack of correspondance between A_1 and D cells has been demonstrated (*Bjorkman et al* 1966 *Hellerstrom & Asplund* 1966)

Many authors have postulated that the argyrophil islet cells demonstrated by the modified Davenport method may constitute a functional unit (*Epple* 1963 *Hellerstrom et al* 1964 *Solcia & Sampietro* 1965) Their morphological similarities with the argyrophil cells in the pyloric antrum have evoked the idea that the argyrophil islet cells produce gastrin and are the origin of the Zollinger Ellison tumours from which

also a gastrin like substance has been isolated (*Zollinger et al* 1962 *Jackson et al* 1963 *Gregory & Tracy* 1964 c *Cavallero & Soleia* 1965) The present work provided no evidence of regressive changes of the A_1 cell nuclei after 5 days treatment with a substance isolated according to *Gregory & Tracy* (1964 a) The frequency curves of the A_1 cell nuclei had about the same profile as the A cell nu curves both in the gastrin injected animals and in the controls and were in either case dislocated to the left as compared with the A cell nuclei curves The gastrin doses used in the present work were of about the same size (corresponding to 1 6–3 2 μg per kg body weight) as those stimulating the gastric secretion in dogs for up to 3 hours (*Gregory & Tracy* 1964 b) In evaluating the present result it must however be considered that the dose response in guinea pigs is not exactly known Opinions on the identity of the substance here used and the hormone gastrin are also diverging (*Tauber & Madison* 1965 *Gregory & Tracy* 1966 *Tauber* 1966) Further tests using other substances which display a gastrin like activity and also tests on other animal species must be performed in order to analyse the relationship between the A_1 cells and the Zollinger Ellison tumours It should also be stressed that the tumours may originate from many other cell types such as the atopic antral mucosa cells for example

SUMMARY

A technique to be used for the staining of thin pancreatic sections of Epon embedded material served to provide a detailed morphological evaluation of the A_1 A and B cells in the islets of Langerhans in guinea pigs With a view to testing the hypothesis that the A_1 cells may synthesize gastrin this substance was injected into guinea pigs every third hour for 5 days No qualitative or quantitative changes were found in any of the three types of granulated islet cells

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CYTOPHOTOMETRIC MEASUREMENTS OF THE DNA CONTENT OF LUNG TUMOURS

By

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Received 3 x 67

Fine needle aspiration cytology has come into common use for diagnosing tumours in several organs e g lymph nodes thyroid salivary glands mammary gland the prostate etc (*Soderstrom* 1966 *Esposti et al* 1967) During the last years techniques have been developed at Karolinska Sjukhuset which allow cytologic samples to be withdrawn from all parts of the lungs and mediastinum These techniques have been especially useful when malignant tumours were to be diagnosed and have also proved to be of diagnostic value in cases of benign tumours and inflammatory lesions The criteria for a morphological judgment of such specimens have been standardized and systematized (*Dahlgren Nordenstrom* 1966)

Some recent studies have indicated that diagnostic judgments may be possible on the basis of quantitative measurements of nuclear DNA contents (*Atkin* 1964 *Wied* 1966) However with the exception of a recent work by *Sandritter et al* (1966) where cytophotometric DNA assessment was performed on six lung biopsy specimens there is a dearth of cytophotometric information about lung tumours and none relative to diagnostics as opposed to that available concerning post operative specimens

In this study we have combined our experience concerning the lung puncture technique and the cytological diagnosis of such specimens with quantitative measurements of nuclear DNA We have also whenever possible followed up these determinations with others on surgical or autopsy material In this way we seek to relate the per cell nuclear DNA values and the DNA distribution patterns to the common cytological and histological classification of the same specimens The main purpose of this investigation is to obtain information about the DNA distribution in lung tumours and to find out whether the information obtained is of diagnostic value

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METHODS

Needle Biopsy Technique

With the aid of television fluoroscopy small radiologically detected lesions in the lungs and mediastinum were punctured and biopsy material thus obtained by aspiration The technique used permitted puncturing of and aspiration from lesions as small as 5 mm in diameter located anywhere in the lungs or mediastinum Very small lesions which were difficult to localize were punctured during simultaneous television fluoroscopy in two right angle planes

The biopsy needles usually employed were 9 to 16 cm long with an outer diameter of 0 9 to 1 1 mm The tips were sharp and obliquely ground Each needle had a ground in mandrin which during insertion prevents tissue from entering the needle s lumen Aspirations were performed with ordinary 10 or 20 cc syringes For further information about the aspiration technique and other technical details the reader is referred to *Dahlgren & Nordenström* (1966)

Cytological and Histological Technique

Aspirated biopsy material was smeared on glass slides Slides intended for common cytological evaluation were immediately fixed in a mixture of equal parts of absolute ethanol and ether whereas those prepared for cytophotometric DNA studies were air dried

Fixed slides were stained with haematoxylin eosin according to Harris (*Lillie* 1965) Air dried slides were fixed for ten minutes in chloroform Carnoy s solution and stained according to a modified Feulgen procedure previously described the hydrolysis was carried out for a period of six minutes at 50 C in 1 N HCl (*Adams* 1964 1967)

Cytophotometric studies were also performed on surgical and autopsy specimens In these cases single cell suspensions of excised tumour material were made in one of the following ways (A) If the tumour was soft a pea sized portion of the latter was minced with a razor blade and drawn through successively finer guage hypodermic needles with saline until a single cell suspension was obtained The suspension was then centrifuged to bring the cells down as a soft pellet the supernatant discarded and a volume of calf serum approximately equal to that of the pellet added The cells were resuspended in the calf serum by stirring and this suspension was painted onto microscope slides with a sable brush (B) If the tumour was hard (fibrous) it was scraped with a knife and the mushy mass collected on the knife blade was suspended in saline by shaking The saline suspension was then centrifuged resuspended and placed on the microscope slides as described above In both cases slides were air dried fixed in chloroform Carnoy s solution and Feulgen stained in the same manner as the slides of aspirated material

The surgical or autopsy specimens for histological examination were fixed in 10 per cent neutral formalin and embedded in paraffin Sections were prepared and stained with haematoxylin eosin according to Harris (*Lillie* 1965)

Cytophotometric Method

Cover glasses were mounted on the Feulgen stained slides with mounting oil of refractive index 1 546 (R P Cargille laboratories Inc) As many abnormal cells (*i e* cells which definitely were not inflammatory cells or normal epithelial cells) as could be located on each slide were photomicrographed on Eastman Kodak High Contrast Copy 35 mm film using a Leitz Ortholux microscope fitted with an Orthophot camera and a 25 X NA 0 50 Plan objective The NA 0 95 achromatic condenser was stopped down to approximately NA 0 45 and the microscope s built in tungsten illumination was rendered approximately monochromatic at 560 mμ with a Schott line (interference) filter Exposure settings and film development conditions were chosen on the developed film to obtain a background transmission of about 30 per cent The film was developed in Kindermann tanks with Kodak developing formula D 90 diluted with two parts of water for 7 0 minutes at 20 C (Potassium bromide was omitted from the developer) Under these conditions of staining and photomicrograph the transmission of the lightest portions of the negative nuclear images remains under 70 per cent A previously described linear relationship between the specimen absorbance (which is proportional to the amount

TABLE 1

Material Comprising 10 Cases with Epidermoid Carcinoma (Ec), 6 Cases with adenocarcinoma (Ac) and 8 Cases with Undifferentiated Carcinoma (ca). Cytophotometric Analysis (Cpha) was Performed in all Cases on Lungpuncture Specimens and/or Surgical or Autopsy Specimens

Case no	Age	Sex	No	Lung puncture specimen: Cytological diagnosis	Cpha	No	Surgical or autopsy specimen: Histological diagnosis	Cpha
1	62	♂	C1	Slightly differentiated Ec	+	H1	Slightly differentiated Ec	+
2	57	♂	C2	Moderately differentiated Ec	+	H2	Slightly differentiated Ec	+
3	64	♂			−	H3	Slightly differentiated Ec	+
4	61	♂			−	H4	Moderately differentiated Ec	+
5	6	♂			−	H5	Moderately differentiated Ec	+
6	59	♂			−	H6	Moderately differentiated Ec	+
7	88	♂	C7	Moderately differentiated E	+			−
8	57	♂	C8	Slightly differentiated Ec	+			−
9	73	♂	C9	Moderately differentiated Ec	+			−
10	49	♂	C10	Slightly differentiated Ec	+			−
11	69	♀	C11	Moderately differentiated Ac	+	H11	Moderately differentiated Ac	−
12	16	♂	C12	Slightly differentiated ca	+	H12	Slightly differentiated Ac	+
13	72	♂	C13	Slightly differentiated Ac	+	H13	Slightly differentiated Ac	+
14	62	♂	C14	Moderately differentiated Ac	+	H14	Highly differentiated Ac	−
15	63	♀	C15	Moderately differentiated Ac	+	H15	Slightly differentiated A	
16	44	♀			−	H16	Slightly differentiated Ac	+
17	63	♀	C17	Slightly differentiated ca	+	H17	Small cell anaplastic ca	+
18	81	♀	C18	Small cell anaplastic ca	+	H18	Small cell anaplastic ca	
19	57	♀	C19	Small cell anaplastic ca	+			−
20	63	♂	C20	Small cell anaplastic ca	+	H20	Large cell anaplastic ca	−
21	54	♂			−	H21	Large cell anaplastic ca	+
22	72	♂	C22	Moderately differentiated A	+	H22	Large cell anaplastic ca	−
23	65	♂	C23	Slightly differentiated ca	+			−
24	4	♂	C24	Anaplastic ca	+	H24	Anaplastic ca	−

of bound Feulgen stain) and the transmission of the developed film then allows the direct photometric estimation of DNA per nucleus (*Adams* 1967)

Photometry of the developed nuclear images was performed with the previously described photometer and data collection was facilitated by the use of a semi automatic electromechanical system (*Adams* 1967) The results given below represent the difference ΔT between the transmission of a nuclear image and that of an identical sized background area adjacent to it As shown in earlier publications (*Adams* 1964 1967) the ΔT value is proportional to the total extinction of the Feulgen DNA complex in each nucleus under the prevailing experimental conditions To control the variations in the Feulgen staining procedure a slide of mouse thymus lymphocytes has been stained together with each group of lung puncture smears According to the results there is a good correlation in Feulgen DNA distribution between normal mouse thymocytes and normal human granulocytes The ΔT values for mouse thymus lymphocytes have been taken to indicate the normal diploid post mitotic DNA values

Nature of and Selection of Specimen Material

The tumour specimens can be classified as follows (1) cell material for cytological examination obtained by transthoracic needle biopsy (18 cases) (2) tissue material for histological examination from primary lung tumours obtained during surgery or at autopsy (18 cases) In twelve of these cases it has been possible to obtain specimens of both categories from the same tumour Cytological and/or histological specimens from the total amount of 24 patients have also been cytophotometrically examined The tumour material has been grouped histologically according to *Kreyberg et al* 1967 The tumours have further been evaluated with a view to determining whether the cells were morphologically highly differentiated moderately differentiated, or undifferentiated Table 1 shows the origin of the specimens studied here and also the cytological and the histological classifications made at the time of puncture biopsy surgery or autopsy

To find out whether the DNA distribution differed in the central and peripheral portions of a tumour specimens from different portions have been collected in two cases

In chronic inflammatory lesions the epithelial cells can show marked morphological nuclear changes (*Grunze* 1955 *Koss* 1961) The inflammatory changes can simulate the cell picture of malignant tumours and thus in cases of lung puncture specimens cause diagnostic errors (*Dahlgren* 1967) In order to compare the pattern of DNA distribution in chronic inflammation and that of malignant lung tumours we have studied the DNA distribution in epithelial cells in four cases of proved chronic tuberculosis of the lungs Some of the epithelial cells showed nuclear abnormalities although they were not as marked as those occasionally seen to be responsible for diagnostic errors in cases of tuberculosis (*Dahlgren* 1967)

RESULTS

The results are presented in the form of histograms in Figs 2–7 The histograms show the distribution of the Feulgen DNA values for each cytologically or histologically prediagnosed sample The tumour cells to be measured were selected after comparison with cells found in the haematoxylin eosin stained slides prepared from the same tumour thus facilitating the selection of tumour cells for Feulgen DNA measurements Since Feulgen stained slides show only nuclear structures it can not be totally excluded however that a few abnormal but not malignant cells might have been included in the tumour cell populations Some Feulgen stained tumour cells selected for cytophotometric analyses are demonstrated in Fig 1 The total number of cells measured

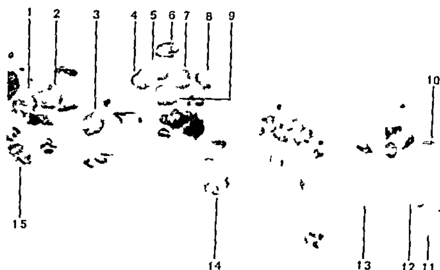


Fig 1

Feulgen stained slide showing how tumour cells have been selected for cytophotometric analyses In this specimen (C14) there is a relatively high content of non tumour elements mainly macrophages In other cases the slides show a more pure tumour cell population Measured cells are indicated 1 to 15

in the populations is given for each specimen (Table 2) When possible 100 cells were assessed for Feulgen DNA measurements

Normal Mouse Thymocytes

The DNA values for the 15 thymus lymphocyte populations used as controls show a fairly symmetrical distribution around the mean values with a small degree of variation (Fig 2) The coefficient of variation (100 × standard deviation/mean) for the DNA values range from 5 to 18 The mean values are taken to indicate the normal diploid post mitotic DNA values These values are used as standards for the interpretation of the other histograms and are designated 1 relative unit

Normal Human Granulocytes

Human granulocytes were studied in lung puncture material from two cases of acute unspecific inflammatory reaction In Fig 2 a comparison between the DNA distribution in mouse thymocytes and human granulocytes is demonstrated The Feulgen DNA histograms for the two human granulocyte populations show a narrow distribution similar to that observed in mouse thymocytes The mean values for the Feulgen DNA values are 1 0 and 0 9 relative units the coefficients of variation being 11 and 10 0 respectively

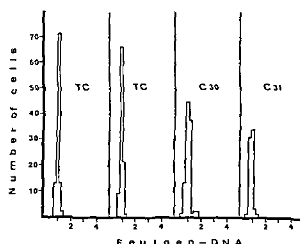


Fig 2 Comparison between Feulgen DNA distribution in two cases of normal mouse thymocytes (TC) and in two cases of normal granulocytes (C30 C31) Diploid post mitotic DNA values for the control thymocytes designate 1 relative unit

TABLE 2

Feulgen DNA Values in 29 Specimens with Malignant Tumours

Case no	Histological or Cytological *diagnosis*		Feulgen DNA values		
	Type of tumour	Degree of differentiation	Mean value (relative units)	Coefficient of variation	Number *of* cells measured
C 1	Epidermoid ca	slight	2 5	56	94
H 1	Epidermoid ca	slight	2 4	49	100
C 2	Epidermoid ca	slight	2 6	77	27
H 2	Epidermoid ca	slight	2 8	68	63
H 3	Epidermoid ca	slight	1 5	53	100
H 4	Epidermoid ca	moderate	1 7	43	100
H 5	Epidermoid ca	moderate	1 5	31	70
H 6	Epidermoid ca	moderate	2 3	44	55
C 7	*Epidermoid ca*	*moderate*	3 6	61	59
C 8	Epidermoid ca	slight	2 8	45	75
C 9	Epidermoid ca	moderate	2 2	40	75
C 10	Epidermoid ca	slight	1 7	37	100
C 11	Adenocarcinoma	moderate	2 2	42	90
C 12	Adenocarcinoma	slight	2 5	36	100
H 12	*Adenocarcinoma*	slight	2 9	38	7[illegible]
C 13	Adenocarcinoma	slight	2 7	33	83
H 13	Adenocarcinoma	slight	2 9	32	100
C 14	Adenocarcinoma	high	3 5	35	90
C 15	Adenocarcinoma	slight	5 0	18	88
H 16	Adenocarcinoma	slight	1 5	37	97
C 17	Small cell ca	anaplastic	1 5	31	100
H 17	Small cell ca	anaplastic	1 2	47	100
C 18	Small cell ca	anaplastic	2 0	45	100
C 19	Small cell ca	anaplastic	1 8	21	93
C 20	Small cell ca	anaplastic	2 6	29	100
H 21	Large cell ca	anaplastic	1 [illegible]	29	100
C 22	Large cell ca	anaplastic	1 6	32	98
C 23	Ca	anaplastic	2 4	41	100
C 24	Ca	anaplastic	2 0	28	100

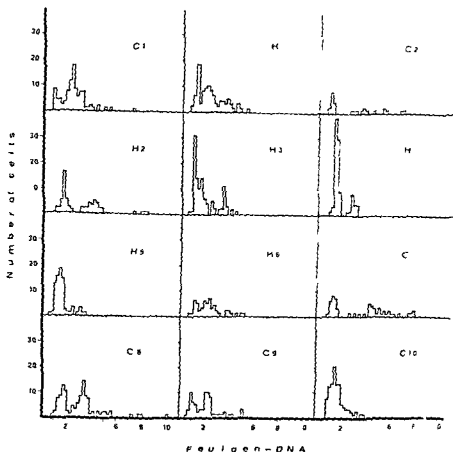


Fig 3

Histograms of the Feulgen DNA distribution in the ten cases of epidermoid carcinoma Diploid post mitotic DNA values for control thymocytes designate 1 relative unit

Tumour Cell Populations

As shown by the histograms there is a marked difference between the DNA distribution of normal human granulocyte and normal mouse thymocyte populations (Fig 2) and that of tumour cell populations (Figs 3–5) The tumour cases have a wider variation in their DNA distribution patterns The mean values of the total Feulgen DNA values in tumour cell populations are significantly higher than the corresponding values for those of normal cells In Table 2 the mean value and the coefficient of variation in the individual cases are summarized The mean value of the Feulgen DNA values for the total number of tumour cell populations is 2 3 relative units

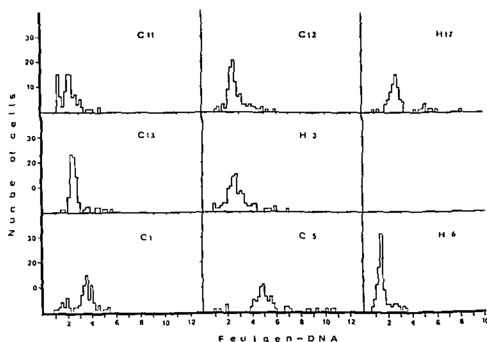


Fig 4

Histograms of the Feulgen DNA distribution in the eight cases of adenocarcinoma Diploid post mitotic DNA values for control thymocytes designate 1 relative unit

Epidermoid Carcinoma

When the tumour material is classified and grouped according to histological or cytological type of tumour the mean Feulgen DNA value in the group of epidermoid carcinoma is found to be 2 3 relative units As apparent from the histograms in Fig 3 and the values for the coefficient of variation in Table 2 the degree of variation in the Feulgen DNA values is high in the case of the epidermoid carcinoma the coefficient of variation ranging from 31 to 77 The histograms demonstrate some bimodal distribution of the values the peaks ranging between 1 0 to 2 0 relative units and around 2 5 to 3 5 relative units

Adenocarcinoma

In cases of adenocarcinoma there is a tendency to exhibit Feulgen DNA values higher than those seen in cases of epidermoid carcinoma (Fig 4) This difference however is not statistically significant The mean Feulgen DNA value in the group of adenocarcinoma is 2 9 relative units The adenocarcinoma histograms characteristically have the DNA values in one fairly tall peak In most of the samples this peak is found between 2 0 and 3 0 relative units In two cases (C14 C15) ... elative units respectively

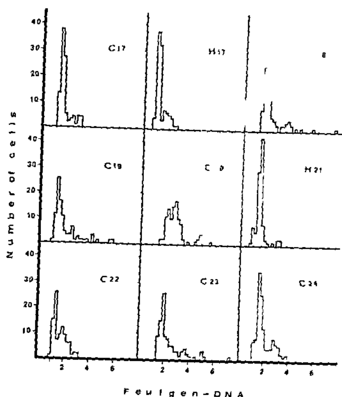


Fig 5

Histograms of the Feulgen DNA distribution in the nine cases of undifferer te l carcinoma. Diploid post mitotic DNA values for control thymocytes desig te 1 relative unit

In cases of adenocarcinoma there is a tendency for the Feulgen DNA values to spread symmetrically around the demonstrated peak. The coefficient of variation for the group of adenocarcinoma ranges from 18 to 42

Undifferentiated Carcinoma

In undifferentiated carcinoma the mean Feulgen DNA value is 1.8 relative units. Undifferentiated carcinoma have a slight tendency to show DNA values that are lower than those observed in cases of epidermoid carcinoma and adenocarcinoma. These findings could however only be proved with some significance (0.01<P>0.002) if the values were compared with those obtained in cases of adenocarcinoma. As demonstrated in the histograms in Fig. 5 the Feulgen DNA values in undifferentiated carcinoma show one narrow peak situated between 1.0 to 2.0 relative units in eight cases and between 2.0 to 3.0 relative units in one case. The coefficient of variation ranges from 21 to 47

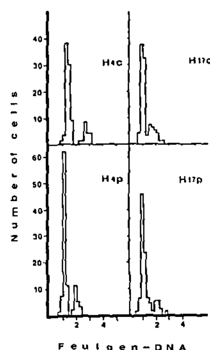


Fig 6 Histograms of the Feulgen DNA distribution in cells from the central (c) and peripheral (p) portions of one epidermoid carcinoma (H4) and one small cell anaplastic carcinoma (H17) Diploid post mitotic DNA values for control thymocytes designate 1 relative unit

Comparison between Central and 'Peripheral Tumour Cell Populations

The histograms in Fig 6 demonstrate the distribution curves obtained in two cases in which it was possible to study the Feulgen DNA values in cells from different portions of a tumour The distribution of Feulgen DNA values in central and peripheral portions of the tumours were not found to differ One case was a centrally situated epidermoid carcinoma (3 cm in diameter) the other was a small cell anaplastic carcinoma (oat cell type) centrally situated and measuring about 10 cm in diameter

TABLE 3

Feulgen DNA Values in Four Cases of Tuberculosis

Case no	Feulgen DNA values		
	Mean value (relative units)	Coefficient of variation	Number of cells measured
C 26	1 4	53	67
C 27	1 2	20	100
C 28	1 1	41	87
C 29	1 6	35	100

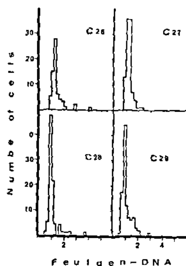


Fig 7 Feulgen DNA distribution in four cases of proved tuberculosis Diploid post mitotic DNA values for control thymocytes designate 1 relative unit

Tuberculosis

The four cases of tuberculosis exhibit fairly narrow distribution curves with a peak near 1 0 relative unit (Fig 7) The mean values and coefficients of variation for these four cases of tuberculosis are demonstrated in Table 3 In this group the average value of the Feulgen DNA mean values is fairly high in cell populations from patients with tuberculosis The mean value for the coefficient of variation is about 37

DISCUSSION

In agreement with earlier cyto chemical *in vivo* studies on human malignant tumours (among others *Moberger* 1954 *Mellors* 1955 *Caspersson* 1964 and *Sandritter et al* 1966) the cytophotometric results obtained in the present study show that the cellular DNA distributions in malignant tumour cell populations of the lung show a great variation of the DNA values above the normal diploid one The difference we observed between neoplastic cell populations on the one hand and normal human granulocytes and mouse thymocytes on the other may depend to some extent on the fact that we have compared proliferating cell populations with non proliferating The difference in Feulgen DNA distribution between tumour cell populations and normal thymocyte granulocyte populations however seems to be too large to depend only on the enhanced proliferation of the neoplastic cells The majority of the tumour cell populations demonstrate Feulgen DNA values clearly above 2 relative units which corresponds to the normal pre mitotic value and indicates the presence of heteroploidy The heteroploid pattern is most evident in the groups of adenocarcinoma and epidermoid carcinoma

According to *in vitro* studies (*Killander* 1966) the presence of abnormal non dividing cells also causes variations in the intercellular DNA distribution In our *in vivo* study it is not possible to determine the stage at which the cell has arrived in the mitotic cycle On the basis of histological and cytological methods however, we can classify the abnormal cells morphologically In epidermoid carcinomas the nuclear size and form are more heterogenous than in adenocarcinomas and undifferentiated carcinomas especially as regards the small cell (oat cell) type It is interesting to notice that the degree of variation in the Feulgen DNA values follows to some extent the morphological classification of the tumours epidermoid carcinoma exhibits the largest degree of DNA differences and the monomorphous oat cell carcinoma show a narrow Feulgen DNA distribution curve The differences in coefficient of variation for the three groups of tumours are however statistically not significant

In the different groups of lung tumours the degree of Feulgen DNA variation changes from one case to another especially in the group of epidermoid carcinoma

In some of the epidermoid carcinomata and the undifferentiated carcinomata a concentration of cells is found at normal diploid DNA values This can to some extent be caused by the fact that a few abnormal but not malignant cells have been measured together with the tumour cells an inevitable error partly due to the difficulty in differentiating certain cells morphologically on Feulgen stained slides Even if there exist some differences in the DNA distribution pattern in different types of tumours it is not possible to differentiate statistically between epidermoid carcinoma and adenocarcinoma of the lung only on the basis of Feulgen DNA measurements The significant differences in mean Feulgen DNA values for adenocarcinoma and undifferentiated carcinoma however may be of diagnostic value

In agreement with *Sandritter et al* (1966) any distinct differences in Feulgen DNA values in samples from the central and the peripheral portions of a tumour are not demonstrated The material studied—two cases—is however too small to allow any definite conclusion to be drawn *Sandritter et al* (1966) suggested on the basis of his findings that cellular synthesis of DNA occurred in all layers of a carcinoma

Whenever abnormal cells found in cases of tuberculosis are measured there are no statistically significant differences in Feulgen DNA values as to the normal thymocyte granulocyte populations The small pattern differences existing can be explained exclusively by the proliferative activity of the cells in the cases of tuberculosis There are significant differences between the Feulgen DNA values for abnormal cells from cases of tuberculosis and those from cases of epidermoid carcinoma and adenocarcinoma It is however of particular interest to compare the histograms in cases of tuberculosis with those of small cell anaplastic carcinoma Some of the histograms in these two groups are of

similar type and show no statistically significant differences These results can to some extent explain the difficulties involved in in interpretation of the morphological pattern of cytological specimens from cases with marked chronic inflammatory reaction

In the five cases studied by cytophotometric analyses of both cytological material and material obtained at surgery or autopsy there is a good correlation in Feulgen DNA distribution patterns (Fig C and H 1 C 2 and H 2 Fig 4 C 12 and H 12 C 13 and H 13 Fig 5 C 17 and H 17) The small differences in distribution patterns can be explained entirely by the fact that the specimens are collected at different times In some of these cases more than three months have elapsed from the first examination to the final examination of surgery or autopsy specimens

The results obtained in this investigation indicate that Feulgen DNA measurements on aspiration biopsy specimens can provide useful information which if added to the subjective morphological criteria can be of diagnostic value As transthoracic needle aspiration biopsy is easily performed and modern technique allows rapid cytophotometric analysis even on large materials the methods can be used together In that way it may be possible to obtain a broader basis of information about the Feulgen DNA distribution in different types of lung tumours and possibly to correlate Feulgen DNA distribution patterns with clinical data Such continued studies may be useful in the future when automatic cytological methods may become a reality

SUMMARY

In order to correlate the per cell nuclear DNA values and the DNA distribution patterns with cytological and histological classification of lung tumours cytophotometric analyses have been performed on 29 specimens from 24 patients The cell material was obtained by fine needle aspirations by surgery or at autopsy Cytophotometric analyses demonstrated a clear difference between mean values and distribution patterns of the Feulgen DNA values obtained from normal inflammatory cells and those obtained from tumour cell populations Differences in the Feulgen DNA values and their distributions also indicate a tendency of certain tumour types to show specific distribution curves

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A METHOD FOR PRODUCING EXPERIMENTAL SKELETAL TUBERCULOSIS IN BONE MARROW NECROSIS IN THE GUINEAPIG

By

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Received [illegible]

The production of skeletal infections in experimental animals has always proved difficult especially chronic skeletal tuberculous lesions in small laboratory animals Attempts to produce such lesions in rabbits or guineapigs (*Trudel* 1933 *Zappio & de Blasio* 1935 *Boquet & Laporte* 1936 *Koch* 1950 *Bastos Mora & Portal* 1953) have been unsuccessful either because the animals died too early from systemic tuberculosis or because the method used failed to produce any skeletal lesion at all Recently however *Lindberg* (1967) described a method which almost invariably produced chronic skeletal tuberculous processes in guineapigs According to this method a porous form of hardened gelatin sponge Spongostan* is placed for one week in a liquid medium inoculated with tubercle bacilli and then deposited in the marrow cavity of the femur through a hole drilled in the bone The animals survive for at least 4 months within which time a typical tuberculous abscess develops in the marrow cavity

Since the production of skeletal infections in experimental animals has proved to offer serious difficulties it was thought worthwhile also to describe another method which will almost invariably induce skeletal tuberculosis in guineapigs The method is based largely on the procedure used by *Scheman et al* (1941 1943) to induce staphylococcal osteomyelitis in rabbits They injected first sodium morrhuate (which tends to produce necrosis and is also used as a sclerosant in the treatment of varicose veins) through a hole drilled in the tibia of rabbits and half an hour later staphylococci through the same hole This procedure invariably produced staphylococcal osteomyelitis In the experiments described below tubercle bacilli were used instead of staphylococci and guineapigs instead of rabbits

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* Manufactured by AB Ferrosan

This investigation was made possible by a grant from Svenska Nationalföreningen mot Hjärt- och Lungsjukdomar [illegible]

MATERIAL AND METHODS

Tubercle Bacilli

Mycobacterium tuberculosis strain H 37 Rv from the Central Bacteriological Laboratory of Stockholm City was used. The bacilli were cultured in the following way

From cultures in Lowenstein-Jensen tubes 10 loopfuls were transferred to a dish homogenized with a glass rod and suspended in 5 ml of physiological saline 0 5 ml of this suspension was transferred to 5 ml of Dubos liquid medium The tube was then incubated for one week at 37 C during which time it was thoroughly shaken once a day After one week 0 5 ml of the culture was transferred to a second tube with 5 ml of Dubos liquid medium. This tube was treated in the same way as the first one and after one week the bacterial culture was used

Operation and Histological Technique

The animals were anaesthetised with mebumal intraperitoneally and a hole was drilled in the lateral distal part of the left femur 0 05 ml of sodium morrhuate was injected through the hole After half an hour 0 05 ml of the bacterial culture containing about 12 5 × 10^6 bacilli, was injected through the hole and the wound was closed. The number of bacilli was counted according to *Mackeprang* (1960) (see Discussion)

The animals were killed after varying intervals the operated leg was removed decalcified in trichloracetic acid and embedded in paraffin Histological sections were prepared and stained with haematoxylin eosin and according to van Gieson.

Animals and Vaccination Procedure

61 guinea pigs weighing about 200 g were used When purchased, and again just before use the animals were examined with intracutaneous injection og 01 ml of tuberculin, diluted 1 100 into the right flank. All animals in which the reaction was equivocal were rejected

A troublesome problem in experimental skeletal tuberculosis is the short duration of survival of the animals owing to massive spread of tubercle bacilli. This problem is serious because a typical skeletal lesion with reactive changes requires a fairly long time to develop The duration of survival can be prolonged by previous vaccination with BCG (*Bogen & Loomis* 1935 *Spiess* 1953 *Griesbach* 1954 *Rosenthal* 1957 *Linlberg* 1967)

According to the same authors immunological factors may also decide the site and location of an infection and experiments carried out by *Lurie* (1939) *Spiess* (1953) *Spiess & Poppe* (1954) and *Strom* (1955) suggest that tubercle bacilli are retained longer at the site of inoculation in vaccinated animals than in unvaccinated ones.

In the present work the experimental animals were vaccinated for two reasons to prolong the duration of survival and to give if possible a better location of the infection to the site of inoculation in the skeletal focus. The experiments were carried out on the following 4 groups of animals

Control group (23 animals) Only morrhuate was used. The animals were killed after 24 hours 1 2 3 4 6 and 8 weeks respectively and the leg into which the morrhuate had been injected was removed In this group the purpose was to find out how extensive the necrosis produced by the morrhuate was and how long a time such lesions required to heal

Vaccination group (the vaccination scheme has been described previously by *Linlberg* 1967) *Group I* (14 animals) Unvaccinated animals were operated upon and sodium morrhuate and tubercle bacilli were injected into the bone in the way described above The unvaccinated animals in this series were kept untreated until the vaccinations of the animals in groups II and III had been concluded so that the animals in all series were at the same age at the time of operation. The intervals between the operation and death or sacrifice of the animals are given in the table on page 577

Group II (15 animals) was made up of animals vaccinated subcutaneously with 0 1 mg BCG wet weight (from Statens BCG laboratorium Gothenburg) three times at one week intervals Two months after this vaccination had been concluded intra

All tuberculin was received in 1959 and 1960 from the National Bacteriological Laboratory Stockholm

TABLE 1

Survey of Animals

Interval in weeks between operation and sacrifice	1	2	4	6	8	1[illegible]	[illegible]	[illegible]	Total
Group I	1	2	0 (?)	2	[illegible]	[illegible]	[illegible]	1	1[illegible]
Group II	2	?	0 (1)	1 (1)	[illegible]	[illegible]	–	–	15
Group III	1	2	2 (1)	1 (2)	[illegible]	[illegible]	[illegible]	–	1[illegible]

Numerals in brackets denote number [illegible] d l

cutaneous injection of 0 1 ml of tuberculin 1 [illegible] a p [illegible] l l n ing with erythema about 10 mm in diamete[illegible] ulin [illegible] e had been read the animals were operated upon a [illegible] uberc[illegible] [illegible] injected into the bone in the way described [illegible] be we[illegible] uc t or sacrifice of the animals are given in the tab[illegible]

Group III (19 animals) consisted of anima[illegible] at on[illegible] nter[illegible] [illegible]s with a total dose of 30 mg of BCG wet [illegible] Freund [illegible] an One month after the end of the vaccinati[illegible] ntracut[illegible] tion [illegible] tuberculin 1 100 produced a strong local re[illegible]ct[illegible]n with necr[illegible]s Wass[illegible] had also reported such a strong reaction Aft[illegible] the last tuberculin reacti[illegible] read the animals were operated upon [illegible] mor[illegible]huate was injected and tub[illegible] deposited in the way described above The anim[illegible]ls died or were [illegible]acrifice[illegible] intervals after the operation (Table page 57[illegible]

RESULTS

Control Groups

After 24 hours an irregular area of necrotic bone marrow an[illegible] morrhages was seen at the site of the injection This area con[illegible] scattered polynucleated leucocytes

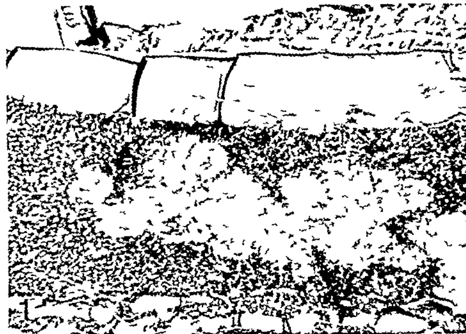
After 1 week the margin of the necrotic focus was bein[illegible] repla[illegible] by granulation tissue which had formed osteoid callus in some areas [illegible]some small necrotic bone trabeculae were seen

After 2 3 and 4 weeks the bone marrow at the site of operation showed a small area with sclerotic bone trabeculae as the only trace of the operation and injection of sodium morrhuate The bone marrow between these trabeculae was normal

After 6 and 8 weeks the whole marrow cavity was of normal appearance

Comment According to *Scheman and co workers* (1943) an injection 0 3–0 4 ml of 5 per cent sodium morrhuate solution into the bone marrow of the tibia of the rabbit produces local necrosis of the bone marrow and of some of the bone trabeculae The lesion begins to heal after one week and after 3 weeks "reparative changes are well advanced

In the present experiments the results were largely similar present ing local necrosis of a small area of the bone marrow and a few bone trabeculae of the lower femoral diaphysis After one week the necrotic



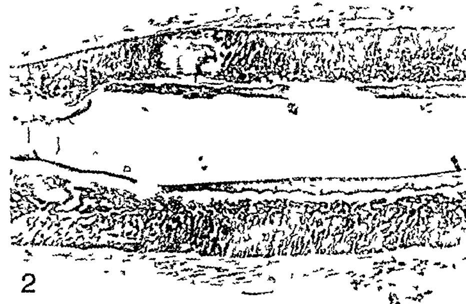


Fig 1 Productive tuberculous focus 1 month after operation Group I van Gieson 70 ×

Fig 2 Total necrosis of marrow and compact bone surrounded by newformed periosteal bone Group I van Gieson 65 ×

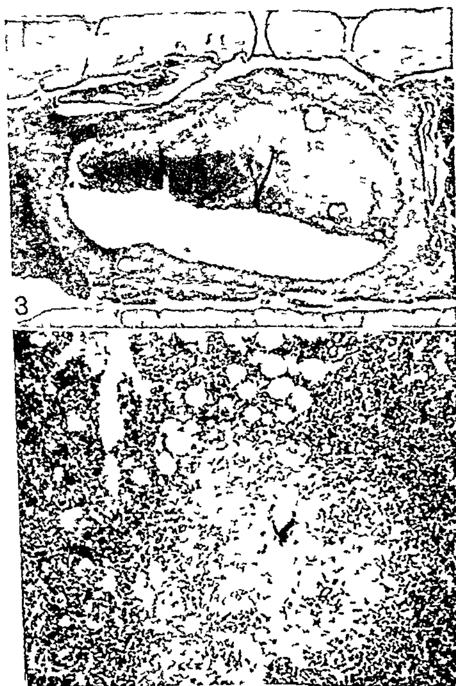


Fig 3 Al sces 4 n tl ft peration Croup I Htx eo in -0 ×
Fig 4 Miliar f ı (w k fter operation Group II Htx eosin 1/0 ×

focus was partly replaced by granulation tissue with some osteoid trabeculae The granulation tissue was replaced by sclerotic callus which was rapidly resorbed and after about 4 weeks completely replaced by normal bone marrow

Group I

After *1–2 weeks* the bone marrow at the site of operation showed an area of granulation tissue with small scattered groups of epithelioid cells and Langhans cells These areas increased in size and within *1 month* they developed into large productive tuberculous foci (Fig 1) scattered in the marrow After *6 and 8 weeks* these foci were still seen

After *12 weeks–6 months* the picture varied from animal to animal In one animal (12 weeks) both the diaphyseal marrow and the compacta had become necrotic and an involucrum had formed (Fig 2) In one animal examined 4 months after the operation a circumscribed abscess had formed (Fig 3) In 3 animals (12 weeks 5 and 6 months) there were productive foci of varying size In one animal (4 months) no tuberculous lesions could be found

Group II

After *1–2 weeks* as in Group I the marrow cavity at the site of operation showed an area of granulation tissue with epithelioid cells But the further development of tuberculosis in this group differed clearly from that in group I After *1 months–6 weeks* the animals had developed only small miliary foci (Fig 4) in the marrow and after 2–4 *months* all the preparations showed a distinct tendency to heal except in one animal in which large productive foci with central necrosis were scattered in the marrow In the other 7 animals large parts of the marrow cavity contained more or less sclerotic bone trabeculae indicating the previous presence of tuberculous foci (*Lindberg* 1967) Three of them showed no signs of tuberculosis while the remaining 4 exhibited small areas of epithelioid cells

Group III

Already after *1–2 weeks* large tuberculous foci (Fig 5) appeared and in one preparation an abscess in the marrow cavity After *1 months–6 weeks* however there was as in group II a clear tendency to healing the foci at the site of the operation often being surrounded by new formed bone (Figs 6 and 7)

2–4 months after the operation 2 of the legs showed no sign of tuberculosis while the remaining 5 contained small foci of epithelioid cells and some bone trabeculae as the only persistent signs of the lesion After *5 months* none of the 3 bones showed signs of tuberculosis

5

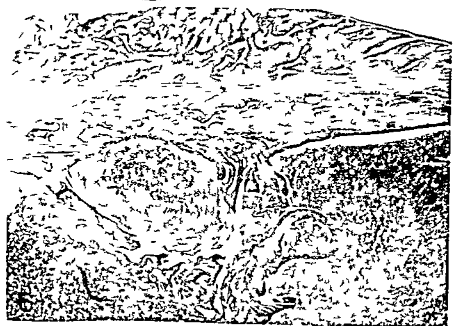


Fig [5] Lesion 2 weeks after operation Group III Htx eosin 85 ×
Fig 6 Lesion surrounded by newformed bone 4 weeks after operation Group III Htx eosin 20 ×



Fig 7 Lesion with epithelioid cells surrounded by newformed bone Observe the well developed margin of osteoblasts Group III Htx eosin 250 ×

DISCUSSION

It is clear from the results set forth above that the infections caused by the method used produced tuberculous lesions in the marrow cavity of most if not all of the animals and that the tendency to healing was strong in the two groups of vaccinated animals

In *group I* the development of the lesions was fairly uniform in all the animals during the first 8 weeks after which the extent of the lesion varied from animal to animal Tuberculous lesions developed in 13 of the 14 animals used

In *groups II and III* the lesions showed a clear tendency to heal already after 1 month and in some of the animals examined 2–5 months after the operation no signs of tuberculosis were seen in the operated femur Tuberculous lesions were found in 12 of the 15 animals in group II and in 14 of the 19 belonging in group III This finding is in accord with previous experience that guineapigs vaccinated with BCG survive longer (*Griesbach* 1951 *Rosenthal* 1957) and that the bone marrow often recovers completely from infections both tuberculous and non tuberculous (*Doan* & *Sabin* 1927 *Mandelstamm* 1933 *Lindberg* 1967) This may explain why it is difficult to produce experimental skeletal infections in animals

In group III large tuberculous foci developed already within 1–2 weeks while groups I and II still showed only smaller areas of granula

tion tissue with scattered epithelioid cells and in some preparations also Langhans cells Also this finding is in agreement with the known fact that a superinfection with tubercle bacilli in a BCG vaccinated animal initially may cause larger tuberculous lesions at the site of inoculation than a corresponding infection in an unvaccinated animal (*Griesbach* 1954 *Rosenthal* 1957 *Lindberg* 1967) The lesions however tend to regress in vaccinated animals but to progress in unvaccinated ones

Out of the 48 animals 17 died spontaneously 4 in group I 5 in group II and 8 in group III (Table 1 on page 577) This mortality is lower than that reported by the aforementioned authors (page 577) but is nevertheless sufficient to limit the practical application of the method The Spongostanmethod described by *Lindberg* (1967) claimed no deaths during the experiments (4 months) and is therefore preferable in this respect

Since the development of the tuberculous lesion varies with the number of bacteria injected attempts have been made to determine the number of bacteria per millilitre of the cultures used by the method of *Mackeprang* (1960) According to this method the aggregates af bacteria in a liquid culture are broken down by ultrasound and the bacteria are counted in stained smears and in culture dilutions grown on solid medium In experiments of the present type finely dispersed cultures of bacteria are unsuitable A culture with large aggregates of bacteria is preferable because the risk of spread of the bacteria from the site of inoculation is probably smaller Therefore an untreated culture was used for injection while the bacterial counts were made in cultures grown at the same time and under the same conditions as the one used

Culture for Bacilli in the Marrow

Since the diagnosis of tuberculosis cannot be considered established if tubercle bacilli in the tissue are not demonstrable marrow specimens from 4 animals operated upon and injected with tubercle bacilli in the manner described above were removed and tested by culture for the presence of tubercle bacilli (*Lindberg* 1967) The animals were not vaccinated and they were killed after 2 4 8 and 12 weeks Tubercle bacilli were found in all of them

Furthermore in histological sections stained according to Ziehl Neelsen a presence of acid fast rods was observed in specimens from the lungs liver and spleen of most of the animals in groups I–III

In sections of the operated femora even in those with histological signs of tuberculosis no acid fast rods could be found It would thus appear that the decalcifying procedure abolished or severely reduced the stainability of the acid fast rods (*Lindberg* 1967)

SUMMARY

A method by which to induce a local tuberculous lesion of the skeleton of the guineapig is described

A small amount of sodium morrhuate is injected into the marrow cavity of the femur A suspension of mycobacterium tuberculosis is then injected into the marrow necrosis produced by the sodium morrhuate A local tuberculous lesion almost invariably develops Most of the animals survive for periods sufficiently long (3–4 months) to permit the lesion to assume a histologically typical appearance and reactions from the surrounding tissue such as newformation of bone

The effect of two forms of vaccination with BCG on the lesions was also studied As regards the duration of survival periods any difference between unvaccinated and vaccinated animals was not observed but the healing tendency of the lesions was stronger in those animals that had been vaccinated most intensely

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CHARACTERIZATION AND PARTIAL PURIFICATION OF STAPHYLOCOCCAL DELTA LYSIN

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Received 18 ix 67

Among staphylococcal toxins those having cytotoxic effects on human cells are of medical interest Out of several extracellular products examined in an investigation described elsewhere (13) delta lysin exerted the most pronounced effect on human erythrocytes and human kidney cell cultures

Relatively little attention has been paid to this toxin since 1947 when it was first described by *Williams & Harper* (28)

A toxin partially purified by means of ethanol extraction was prepared by *Marks et al* (21) and *Jackson et al* (16) In the latter work evidence was presented that delta lysin consists of one heat stable ethanol soluble and one heat labile ethanol insoluble fraction *Hoffmann et al* (14) presented evidence of two haemolysins capable of haemolysing human erythrocytes *Hallander* (10 13) showed that delta lysin eluted at the void volume on Sephadex G 200 well ahead of alpha lysin and beta lysin thus indicating that it is a very large molecule These results were later confirmed by *Kayser et al* (17)

Yoshida (29) obtained delta lysin in crystalline form after chromatography on calcium phosphate gel and triethylaminoethyl cellulose Chemical analysis indicated that delta lysin is a protein with a molecular weight of approx 70000 and a sedimentation constant of 6 1s Crude toxin was completely soluble in chloroform methanol (2 1 v/v) but crystalline delta lysin was insoluble in the same solvent No information was given about the immunological properties Later *Gladstone* reported (7) that these preparations were immunologically heterogenous and contained beta haemolytic as well as RNAase activity

Marks et al (21) and *Jackson et al* (16) found that crude delta lysin was thermostable The crystalline product obtained by Yoshida however lost 85 per cent of its activity upon heating at 100 C for 30 min (29)

Also the antigenic properties of delta lysin have aroused some controversy *McLeod* (22) and *Kayser et al* (17) described the production of neutralizing antibodies by immunization Other workers obtained the same neutralizing capacity with normal serum from rabbit horse man and calf (7 9 15 21)

In the report by *Jackson et al* (15) the Cohn Fractions IV 1 and IV 4 containing mainly alpha and beta globulin were shown to be more inhibitory than the whole globulin fraction *Gladstone* (7) observed marked inhibition with Fraction I (fibrinogen) Fractions III and IV (beta and alpha globulin) and Fraction VI (mucoprotein) Albumin and gamma globulin had little activity

The partially purified product obtained by *Marks et al* (21) was toxic to erythrocytes from a wide range of species These results have been confirmed by *Gladstone et al* (7) and *Hallander et al* (13) for erythrocytes as well as for various cells in tissue culture The toxin is toxic to human leucocytes and has been named leucolysin (6) as distinct from leucocidin Furthermore *Hoffmann et al* (14) reported that partially purified delta lysin demonstrated antibiotic activity against certain gram positive bacteria However no evidence was presented regarding the identity of the antibiotic material(s) and the delta lysins

The capacity to produce delta lysin has been transduced to non haemolytic strains by temperate phages (5)

In this report the purification and some properties of staphylococcal delta lysin are described

MATERIALS AND METHODS

Strains Staphylococcus aureus strain S6 an internationally accepted enterotoxin B producer and 196E, the international prototype strain for enterotoxin A were used for the production of delta lysin The toxin spectra of these strains have been described in detail elsewhere (13)

A strain belonging to the genus of Corynebacterium was used as indicator strain for the assay of bacteriocin like activity

Preparation of crude toxin for fractionation and immunoelectrophoresis For toxin production staphylococci were cultured on solid media covered with cellophane as described earlier (11 21) Crude toxin and pooled fractions were concentrated with polyethyleneglycol (19)

Assay of haemolytic activity 1) The delta lysin titre was routinely measured by incubating 0 5 ml of a 0 5 per cent suspension of washed human erythrocytes group O Rh+ with 0 5 ml of serial dilutions of the sample to be investigated in phosphate buffer saline The titrations were read after 1 hour at 37 C and 1 hour at room temperature The highest toxin dilution showing complete haemolysis was taken as endpoint The reciprocal titre indicates the number of units

2) Erythrocyte membranes prepared according to *Philipson et al* (26) were used for analysis of the time haemolysis curve and the influence of certain variables such as pH cations and EDTA 0 2 ml of delta lysin in phosphate buffer saline ([illegible] titre 1/64) were added to 3 ml of a membrane suspension that had an extinction of 0 3–0 7 in a Beckman Colorimeter type C at a wavelength [illegible] 4 mμ [illegible] filter at 20 C The extinction value was recorded at various intervals

Assay of lipase and alkaline phosphatase was performed as described [illegible] her (10)

Assay of bacteriocin like activity 0 02 ml of each preparation to be [illegible] ed [illegible] dropped on chocolate agar medium together with buffer [illegible] After [illegible] at room temperature for 15 min the dishes were treated with chloroform [illegible] for 15 min and subsequently seeded with a saline suspension [illegible] Readings were made after incubation at 37 C for 24 and 48 hours 4)

Vaccine [illegible] prepared and *immunization* performed as described elsewhere (12)

Gel-diffusion and immunoelectrophoresis were performed as [illegible] described in a previous paper (12) One per cent agarose however was used as a supporting medium

in both methods When haemolytic activity was to be localized the slides were over layed with a 3 per cent solution of washed human erythrocytes in phosphate buffer saline (PBS) After 1 hour at 37 C the slides were rinsed in PBS

Neutralization of delta lysin activity was performed in two ways 1) Sera were titrated in a manner similar to that used in anti staphylolysin titrations (ASTA) by making a two fold serial dilution of a mixture of 0 16 ml of sera inactivated at 56 C for 30 min and 0 84 ml of phosphate buffer saline (PBS) To each dilution was added 0 5 ml of a gel filtered delta lysin (196E) preparation containing 5 hu man haemolytic units After 15 min at 37 C 0 5 ml of 1 5 per cent human red cells was added The titration was read after an additional 30 min at 37 C followed by 18 hours at +4 C

2) Undiluted 0 25 ml samples of the same delta lysin preparations were mixed with equal volumes of serum inactivated for 30 min at 56 C or fractions of this serum After incubation of the mixtures at 37 C for 1 hour they were back titrated for un neutralized delta lysin activity

Trypsin inactivation of delta lysin 0 2 ml of a trypsin solution—0 1 mg/ml in phosphate buffer saline pH 7 2 (PBS) (Worthington Biochemical corp N J U S A 3 × cryst)—was added to 1 8 ml of purified delta lysin pH 7 2 After various inter vals at 37 C 0 1 ml of soybean trypsin inhibitor—0 1 mg/ml in PBS (Sigma Chem Comp Miss U S A 2 × cryst)—was added to 0 4 ml of the delta lysin trypsin mixture After cooling in an ice bath delta lysin activity was determined

As control one per cent suspension of washed human erythrocytes was treated with trypsin at a concentration of 1 mg/ml for 30 min

The protein concentration was determined routinely by measuring the UV absorp tion at 280 mμ in a Beckman DU Spectrophotometer Bovine serum albumin ($A_{280m\mu}^{1\,cm}$ (1 per cent) = 6 6) was used as a reference For recovery studies the solutions to be measured were first adjusted to 0 02 M in sodium phosphate and 0 1 M in sodium chloride pH 7 2–7 4

Gel filtration of toxin preparations was performed on Sephadex G 100 and Sepharose 2 B and 4 B (Pharmacia Fine Chemicals Uppsala Sweden) One glass column 48 × 3 5 cm and one commercial column 45 × 2 5 cm (Type K 25/45 Pharmacia Uppsala Sweden) were used The elution buffer was 0 02 M sodium phosphate con taining 0 1 M NaCl pH 7 2 Elution was carried out at 4 C at 1 5–2 ml/cm /hour and the effluent was collected in fractions of 5 ml

Preparative electrophoresis was performed on a vertical column (50 × 2 2 cm) packed with Sephadex G 25 (Pharmacia Fine Chemicals) void volume 65 ml The column was cooled to 4 with a cooling jacket The material was run towards the anode at 500 V for 24 hours in 0 05 M barbital buffer pH 8 2 In some experiments 0 045 M sodium phosphate buffer pH 6 0 was used Elution was carried out at 4 C at 1 5–2 ml/cm hour The fraction volume was 2 5 ml

Extraction into lipid solvents 1) Evaporated crude toxin—1 g—was extracted into 25 ml of different lipid solvents Twenty ml of the extracts were filtered eva porated once more and then dissolved in the same volume of phosphate buffer saline The delta lysin titres were controlled before and after extraction

2) Non evaporated material usually 1 ml was shaken at room temperature for 30 sec with an equal volume of chloroform methanol (2 1 v/v) The emulsion was centrifuged for 15 min at 4500 r p m The upper and lower phases and the inter faces were evaporated separately and the residues were taken up in the original volume of phosphate buffer saline (PBS)

Phenol extraction was performed according to Goebel et al (8)

RESULTS

Purification Procedure

Delta lysin was purified in a two step procedure involving gel filtra tion and preparative electrophoresis Different approaches to a third step were tried Delta lysin was purified from crude toxin of strain S6 as well as 196E

Step 1 Ten fold concentrated crude toxin was gel filtered through

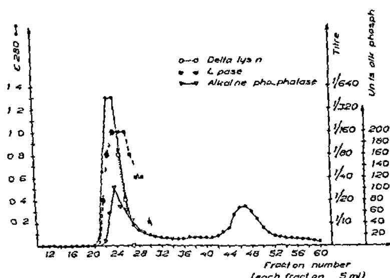


Fig 1

Gel filtration of 10 ml crude toxin from strain S6 through Sephadex G 100 in a glass column 48 × 3 5 cm The elution buffer was 0 0? M sodium phosphate buffer containing *0 1 M NaCl pH 7 ? Elution* was carried out at 4 C at 1 5–2 ml/cm /hour

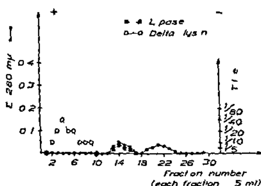


Fig 2

Preparative electrophoresis of 2 ml pooled and concentrated material from the first *peak of the Sephadex G 100 step The electrophoresis was run against the anode* t 500 V in 0 05 M barbital buffer pH 8 2 for 24 hours

Sephadex G 100 As shown previously (10) delta lysin and alkaline phosphatase elute at the void volume together with the lipase An elution pattern typical of strain S6 toxins is shown in Fig 1 Delta lysin from 196L eluted similarly This latter strain produces no lipase The fractions with the highest delta lysin titres were further fractionated

Step 2 Delta lysin material from step 1 was po d ncent at d dialysed against the barbital buffer and subsequently purified further

by preparative electrophoresis The delta lysin from strains S6 and 196L were both negatively charged at pH 8 2 and migrated towards the anode Fig 2 shows a good pattern for S6 delta lysin The toxin is completely separated from the more slowly migrating lipase In some experiments tailing occurred and the lipase fractions were thus contaminated with delta lysin However even in Fig 2 delta lysin activity is seen to be spread over a relatively broad zone (fractions 2–9) Alkaline phosphatase activity could not be recovered after electrophoresis When barbital buffer was replaced by 0 045 M sodium phosphate buffer pH 6 0 the positions of delta lysin and lipase were reversed

Delta lysin from strain 196E migrated at a lower rate (about 10 fractions later) than that of S6

Recovery of Purified Delta Lysin

The recoveries of S6 toxin obtained at different steps in a typical experiment are given in Table 1 Fifty per cent of the original activity was lost during gel filtration 17 per cent during concentration and 12 per cent during electrophoresis The total yield was 21 per cent with a ten fold increase in specific activity Similar results were obtained with delta lysin from strain 196E It is not clear why the specific activity is higher in the unconcentrated than in the concentrated Sephadex peak

TABLE 1
Recoveries of Delta Lysin in the Purification Procedure (S6)

Stage of purification	Volume ml	Delta lysin units Total	Delta lysin recovery between each step %	Overall delta lysin recovery %	Protein γ Total	Specific activity U/γ
Crude toxin centrifuged concentrated	10	6400			850 000	0 008
Unconcentrated Sephadex peak	40	3 200	50	50	40 000	0 08
Concentrated Sephadex peak	6 6	2112	66	33	42 900	0 05
Prep elphores peak	20	1330	63	21	15 510	0 09

Before the protein content was measured all fractions were dialysed against 0 02 M sodium phosphate buffer containing 0 1 M sodium chloride pH 7 2–7 4

Attempts at Further Purification of Delta Lysin

1) *Effect of extraction with lipid solvents* To evaluate the efficiency of lipid solvents crude evaporated toxin from strain 196E was extracted with various lipid solvents Out of the total [illegible] th[illegible] was recovered when the residue was re dissolved in dis[illegible]

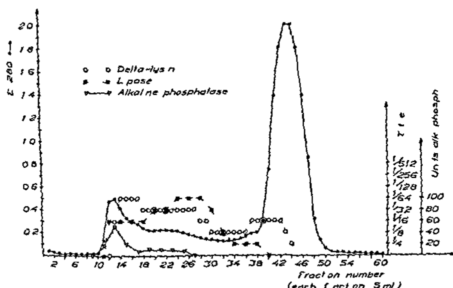


Fig 3

Gel filtration of 2 8 ml crude toxin (strain S6) through Sepharose 4 B in a K 25/45 column. The elution buffer was 0 02 M sodium phosphate buffer containing 0 1 M NaCl pH 7 2 Elution was carried out at 4 C at 1 5–2 ml/cm²/hour

cent was extracted by methanol or chloroform methanol (2 1) 2 5 per cent by ethanol 0 5 per cent by butanol and less than 0 3 per cent by chloroform carbon tetrachloride ether benzene and hexane

Therefore the most active fractions from step 2 were further purified by shaking with an equal volume of chloroform methanol (2 1) With material from both strains a white precipitate was formed at the interface S6 delta lysin activity was evenly distributed between the water phase and the interface The total recovery was 20 per cent This was in contrast to 196E delta lysin most of which appeared at the interface Out of a total recovery of 15 per cent no more than about 1/32 was present in the water phase When the active preparations from step 2 were freeze dried before extraction the recovery was about the same

2) *Phenol extraction* Phenol extraction of material from step 2 abolished all activity

Studies on Size and Homogeneity of Delta Lysin

Since delta lysin did not penetrate the matrix of the Sephadex G 200 gel (10) separation was tried on the more porous Sepharose 4 B and 2 B gels The exclusion limits of Sepharose 4 B (4 per cent agarose) and 2 B (2 per cent agarose) were estimated at 3–4×10⁶ and 20 30×10⁶ expressed as molecular weight of dextran (25) Fractionation of crude S6 toxin on a column packed with Sepharose 4 B is illustrated in Fig 3

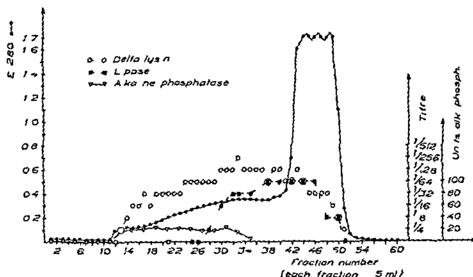


Fig 4

Gel filtration of 4 ml crude toxin (strain S6) through Sepharose 2 B in a K 25/45 column The elution buffer was 0 02 M sodium phosphate buffer containing 0 1 M NaCl pH 7 2 Elution was carried out at 4 C at 1 5–2 ml/cm /hour

Part of the material still eluted at the void volume but the distribution is rather broad and a large retarded peak is present The delta lysin activity is spread throughout The lipase activity closely followed the delta lysin activity but alkaline phosphatase was mainly recovered at the void volume The same distribution was obtained with delta lysin from strain 196E

On Sepharose 2 B S6 delta lysin activity distributed over a very broad zone (Fig 4) encompassing both the alkaline phosphatase which eluted mainly in early fractions and the lipase which eluted later A similar elution curve was obtained with 196E toxin Delta lysin purified by Sephadex G 100 and preparative electrophoresis gave the same activity pattern as the crude material when run on Sepharose 4 B

Recoveries from both Sepharose gels varied between 20 and 30 per cent To increase the recovery 0 05 per cent Tween 80 was added to the buffer (2) A separation of S6 toxin on Sepharose 4 B in the presence of Tween is shown in Fig 5 The total recovery amounted to 120 per cent Delta lysin penetrated the matrix of the agarose gel and was separated into two peaks most of the lipase activity being associated with the first peak Alkaline phosphatase still eluted close to the void volume There was no change in pH during the experiment

Immunological Studies

The purification procedure was monitored immunologically Fractions from various stages of the purification procedure were used for

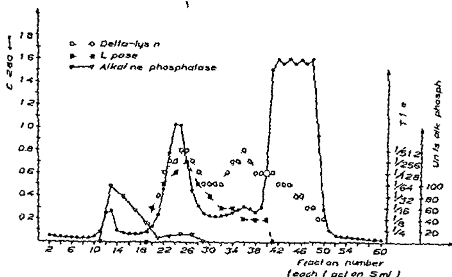


Fig 5

Gel filtration of 4 ml crude toxin (strain S6) through Sepharose 4 B in a K 25/45 column The elution buffer was 0 02 M sodium phosphate buffer containing 0 1 M NaCl pH 7 2 and 0 05 per cent tween 80 Elution was carried out at 4 C at 1 5-2 ml/cm²/hour

immunization All sera were tested by immunoelectrophoresis using crude toxin as antigen Figs 6a and 6b show three precipitation arcs after step 1 and two precipitation arcs after step 2 Precipitation arcs that are present also in the pre immune sera are drawn with broken lines The massive arc in Fig 6a probably consists of several arcs including normal rabbit antibodies to crude toxin When animals were immunized with material obtained after cloroform methanol and phenol extraction no precipitation arcs were obtained with the immune sera The toxins from strains S6 and 196L gave similar results

When delta lysin from the second purification step was used as antigen one long precipitation arc developed with antiserum against delta lysin prepared in two steps (Fig 7b) No precipitation arc developed with pre immune serum The localization of the precipitation arc corresponded to the zone of haemolysis observed after flooding the immunoelectrophoretic slide with human red blood cells (Figs 7a and b)

However the identity of this arc and delta lysin cannot be unequivocally established since the tests used failed to disclose a neutralizing capacity in immune serum compared with pre immune serum Normal serum itself had a certain neutralizing capacity Neither was there any rise in neutralizing capacity of anti crude toxin compared with its pre immune serum though the number of antistaphylolysin units (anti alpha) rose from 0 8 to 6 4

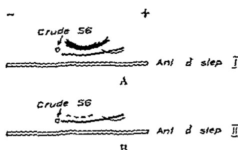


Fig 6 a and 6 b
Schematic representation of immunoelectrophoretic analysis of crude toxin developed with a rabbit serum against step 1 (6 a) and step 2 (6 b) material The broken line represents normal rabbit antibodies to crude toxin

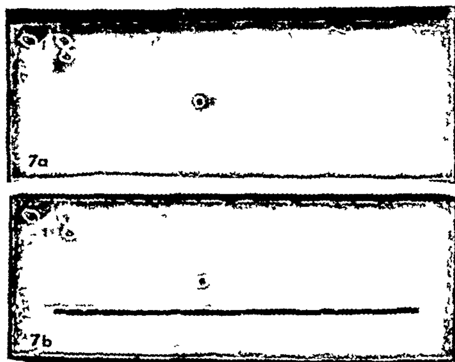


Fig 7 a and b
Electrophoretic analysis of S6 delta lysin purified in two steps The localization of the toxin was revealed with 3 per cent washed human erythrocytes
The same slides as Fig 7 a developed with antidelta serum prepared against delta lysin purified in two steps

Time-Haemolysis Relationships Effect of Various Factors

As demonstrated in Fig 8 delta lysin lysed red blood cell membranes at a high rate Already after 10 sec or as fast as reading was possible 70–80 per cent of the total effect was registered

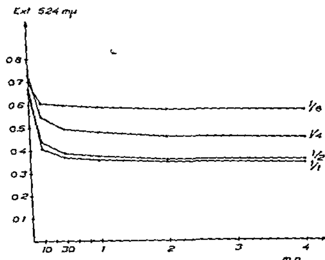


Fig 8

Time haemolysis relationships for erythrocyte membranes and different lysin concentrations The undiluted toxin had a haemolytic titre of 1/64 before it was mixed with the membranes

The effect was studied in 10 ²M solutions of various buffers in the pH range 3 0–10 0 with 0 5 step intervals Any differences in activity were not observed Neither was the activity influenced by the addition of 10 ³M EDTA or 10 ³M MgCl ZnCl CoCl or CaCl in 10 M sodium phosphate buffer pH 7 4

Heat Stability

Purified delta lysin from both strains was completely impervious to boiling for 30 min The delta lysin recovered from the water phase or interface after chloroform methanol extraction was also found to be heat resistant

Trypsin Inactivation

When delta lysin titre 1/160 was exposed to trypsin all activity was lost within 5 min Active delta lysin lysed untreated and trypsin tr t d human erythrocytes equally well

Bacteriocin like Activity

In additi n to the haemolytic effect delta lysin showed a bacteriocin like activity (Table 2 and Fig 9) which however w much weaker than the ha molytic activity 196E toxin seemed to b somewh t more potent th n Sb toxin which never gave complete lysis of the test strain used Durin the purification procedure 196E bacteriocin like activity

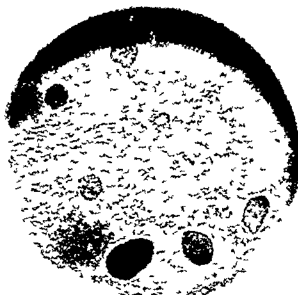


Fig 9

Bacteriocin like activity of 196E delta lysin purified to various degrees From below crude toxin step I and step II material in dilutions of 1/1 1/2 and 1/4

closely followed the haemolytic activity In the case of the S6 toxin the very weak antibiotic effect disappeared already in step 2 The bacteriocin like activity was as heat stable as delta lysin and was also inactivated by trypsin

TABLE 2

Bacteriocin Like Activity of S6 and 196E Delta Lysin Purified to Various Degrees

	Type of material	Haemolytic activity titre	Bacteriocin like activity titre
	Crude toxin	1/2048	(1/1)
	Step 1	1/2048	(1/1)
	Step 2	1/2048	–
S6	CM (2/1) extracted		
	over phase	1/160	–
	interface	1/160	–
	Crude toxin	1/8192	1/4
	Step 1	1/1024	1/2
196E	Step 2	1/1024	1/2
	CM (2/1) extracted		
	over phase	1/8	–
	interface	1/250	–

DISCUSSION

Biologically delta lysin is characterized by its cytotoxic effect on a wide range of species including human cells (7 13 21)

In the present study delta lysin from two strains—S6 and 196E—were studied separately and purified primarily by gel filtration on Sephadex G 100 and preparative electrophoresis the total recovery amounting to about 20 per cent As shown earlier (10 3) delta lysin elutes at the void volume on Sephadex G 100 and is completely separated from *inter alia* alpha and beta lysin

Delta lysin from both strains was also partly excluded by 2 per cent as well as 4 per cent agarose which would suggest a molecular weight of several millions This is not in agreement with the results obtained by *Yoshida* who estimated the molecular weight of delta lysin at approx 70000 (29)

The S6 toxin differed electrophoretically from 196E toxin in that it was more negatively charged at pH 8 2 The difference was emphasized when purified materials were further extracted with chloroform methanol according to *Yoshida* (29) Delta lysin produced by strain S6 was evenly distributed between the water phase and the interface while most of the delta lysin produced by strain 196E collected at the interface

However in addition to the differences between the two delta lysins studied each toxin also appeared to be heterogeneous in character This was indicated by gel filtration on agarose columns On Sepharose 4 B delta lysin from both strains eluted from the void volume to nearly the total volume of the column The marked tailing in electrophoresis also points to heterogeneity After analysis by paper chromatography *Hoffmann et al* (14) suggested that two substances might be present delta A and delta B which were capable of haemolysing human red blood cells *Jackson et al* (16) described one heat stable ethanol soluble and one heat labile ethanol insoluble fraction

On Sepharose 4 B delta lysin entered the gel in the presence of Tween 80 possibly because of de aggregation Tween 80 is a substrate for the lipase present in S6 toxin but not in 196E toxin In the absence of Ca^{++} ions (27) however lipase activity is very low at 4 C results were similar with S6 and 196E toxins

From the immunological experiments it is not possible to draw any final conclusions as regards the antigenicity of delta lysin With the methods used there was however no rise in neutralizing capacity which is in accordance with the results obtained by some other workers (7 9 15 21) This suggests a low antigenicity but the problem will be the subject of further investigation

The heat stability of delta lysin is also controversial *Marks et al* (21) heated crude toxin at 100 for 2 hours and *Gladstone et al* (6) heated it at 115 for 20 min but the change in titre was small only

One of the two fractions described by *Jackson et al* (16) was stable at 60 C The crystalline toxin obtained by *Yoshida* (29) lost most of its activity after 30 min at 100 C In the present study delta lysin was resistant to boiling for 30 min even after extraction with chloroform methanol

The very rapid lytic effect of the toxin agrees with the findings obtained by *Jackson* (15) but the minimum of activity found by this author in the pH range 6 5–7 3 could not be confirmed Neither EDTA nor heavy metal ions had any detectable effect on the lytic activity

There is general agreement concerning the sensitivity to trypsin (17 29) However that delta lysin should be mainly protein is contradicted by the large molecular size combined with the low antigenicity the solubility in chloroform methanol and possibly also by the heat stability In this connection however the bacteriocin like activity is very interesting since all the properties of delta lysin correspond to those of other bacteriocins and bacteriocin like substances (4) It was about the same in both strains Many authors have demonstrated the bacteriocidal activity of staphylococcus *Lachowitz* (20) isolated and described staphylococcin A which was used for local treatment of patients suffering from staphylococcal lesions In 42 cases the treatment lead to complete recovery Staphylococcin A was not destroyed by trypsin but evidently more than one type of staphylococcin may exist since *Barrow* (1) has described an antibiotic substance obtained from Staphylococcus aureus phage type 71 that was inactivated by trypsin Partly purified preparations of delta lysin have also been found to be associated with an antibiotic activity against certain genera of bacteria (14) although the identity with delta lysin could not be established In the present study the staphylococcin of strain 196L was present in the purified delta lysin preparations whereas S6 staphylococcin was lost in step 2 but the antibiotic effect was very weak even before preparative electrophoresis

Colicin K has been shown to be a lipo polysaccharide protein complex (8) When phenol was used to split this complex the colicin activity appeared in the phenol phase but it could not be completely separated from the lipo polysaccharide without loss of activity In this work delta lysin was inactivated by phenol

According to *Ozaki et al* (24) megacin A was found to be phospholipase A that was activated by Ca^{++} ions and inactivated by EDTA Megacin A therefore seems to be unrelated to delta lysin Strains S6 and 196L produce no phospholipase A as shown by *Nygren et al* (23)

The combination of antibiotic and haemolytic activity has also been suggested in the case of enterocin 1 (3) a substance that resembles delta lysin as regards heat stability trypsin sensitivity and low antigenicity

The identity of the haemolytic and bacteriocin like activities will

probably be difficult to prove definitely because of the great difference in sensitivity between erythrocytes and bacteria

SUMMARY

Staphylococcal delta lysins from strains S6 and 196E were purified by gel filtration on Sephadex G 100 followed by preparative electrophoresis and a total recovery of approx 20 per cent Attempts at a further purification included extraction with chloroform methanol (2 1 v/v) and phenol

Sera obtained after immunization with the purified preparations developed a precipitation arc in immunoelectrophoresis the arc was closely related to the zone of haemolysis observed after red cells were added to the plates However there was no rise in neutralizing capacity

In gel filtration delta lysin appeared to be macromolecular with a *heterogeneous and complex structure* Purified delta lysin lysed human erythrocyte membranes at a high rate it was not affected by pH EDTA or cations but it was inactivated by trypsin It resisted boiling for 30 min

As the delta lysin activity accompanied a *bacteriocin like activity* throughout the purification procedures the two activities are probably associated with the same or very similar molecules

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SERUM Pi TYPES IN SOME EUROPEAN, AMERICAN, ASIAN AND AFRICAN POPULATIONS

By

MAGNE K FAGERHOL and OLE W TENFJORD

Received 8 ix 67

The serum alpha 1 antitrypsin has been shown to occur in a surprisingly high number of genetic variants when a special starch gel electrophoretic technique is employed (1 4) These variants constitute the Pi system (Pi is short for protease inhibitor) and to date the following codominant alleles have been described Pi^F Pi^I Pi^M Pi^S Pi^V Pi^X and Pi^Z (1 2 3) The alleles have been given symbols according to the relative electrophoretic mobility of the allele products which decrease in the mentioned order The following phenotypes have been described FF FM FS FZ IM IS MM MS MV MX MZ SS SZ XZ and ZZ (4)

The last mentioned type is associated with a high risk of severe pulmonary disease often resulting in death at about 50 years of age (5)

The studies reported here were made to examine the distribution of Pi types in some non Norwegian populations To detect all the Pi phenotypes a special starch gel technique has to be used This technique has previously only been applied on sera from Norwegians

MATERIALS AND METHODS

As part of a routine health control our laboratory receives blood samples for syphilis antibody screening from most seamen hired on Norwegian ships in Norwegian ports Sera from all foreign seamen hired during one year were selected for this study The samples were coded by serial numbers and st red frozen until te te l After the typings we e complet d the names and addresses of the seamen were disel s d to be gr uped nto the d ffe ent population mples Sera fr m 4? Tibetan refugee boys were also in luded in the material The distributi n of blood gr ups am ng these boys has re ently b en published (6) St r h gel electrophoresis was perf rme l using a horizontal discontinuous system of buffers P tato starch (obtained fr m Jæren Potetmelfabrikk Klepp Norway) was hydrolysed at 44 C f r 7 mins (? ml conc HCl + 600 ml acetone + 300 g starch) The following buffer s luti ns were used anodic e el ?3 5 g citric ac d + 31 4 g Na_2HPO $2H_2O$ aqua de t ad 2 litres

The author thank Mrs *Jorunn Hasli Jacobsen* f r skilful technic l assistance

Cathodic vessel 18.5 g H_3BO_3 + 6 g NaOH aqua dest. ad 2 litres. These buffers may be used for 20–30 runs. Stock solution for preparation of gels: 21 g citric acid + 23 g tris (hydroxymethyl) amino methane aqua dest. ad 1 litre. 200 ml of gel was prepared by heating over an open flame 24 g of starch suspended in a buffer made by adding 185 ml of aqua dest. to 15 ml of the stock solution. The gel was poured into a tray measuring 22 × 12 × 0.4 cm covered by a glass plate and allowed to harden for 30 mins. at room temp. and for one hour at +4° C. Pieces of filter paper dipped in serum were inserted into a slit cut in the gel 8 cm from the cathodic end. Twenty samples could easily be run in one gel. The gel was connected to the electrode vessels by filter paper wicks (8 layers) overlapping the gel ends by 2 cm. The electrophoresis was performed with a d.c. of 8–10 V/cm. To prevent evaporation and excessive heating the gel was covered by a sheet of thin transparent plastic and on top of this a layer of water. The runs were completed when the brown buffer zone had reached 10 cm past the insertion line. The sample filter papers were removed when the buffer zone had migrated 4–5 cm. The gel was cut horizontally into two halves and the bottom part stained with amido black 10 B, making sure that the bottom surface of the gel was adequately stained since this surface often gave the most distinct Pi zones for typing. The gel was destained by acetic acid/methanol/water (1 : 5 : 5). The Pi typing is based upon the migration rates and relative staining intensities of the Pi zones (1). As pointed out in (4) this technique will not detect with certainty all cases of phenotype MZ, the Z zones in this phenotype being very weak (about 5–7 per cent of the MM zones). The sera which on repeated testing showed MM patterns with only about 50 per cent of the normal staining intensity were classified as MZ. These are the same criteria as those used for the typing of the individuals in the Norwegian population sample (1). Antigen-antibody crossed electrophoresis was performed as described in (3) to demonstrate quantitative differences among the Pi zones.

RESULTS

In Table 1 is given the occurrence of different *Pi* phenotypes in six population samples according to the country or part of the world where the individuals are living. It is evident that the distribution of Pi phenotypes in Spaniards and Portuguese differ from that in Norwegians. These differences are caused mainly by higher frequencies of the phenotypes MS and SS and lower frequency of FM in Spaniards and higher frequency of MS in Portuguese. When tested by the four fold table the gene Pi^S is significantly more frequent among Spaniards and Portuguese than among Norwegians (P less than 0.0005). The frequency of Pi^F is significantly lower among Spaniards than among Norwegians ($0.01 < P < 0.025$) when tested by the four fold table with Yates correction.

In addition to the individuals listed in Table 1, nine subjects from Algeria and Morocco were examined; they were all of phenotype MM.

The population sample called "Europe except Norway, Spain and Portugal" in Table 1 includes the following number of subjects and phenotypes from Mediterranean countries: France 9 MM, Greece 8 MM, Italy 9 MM, 1 MS, Jugoslavia 17 MM. When this sample comprising individuals from Mediterranean countries is compared with findings in Spaniards, it is found that Pi^S is significantly more frequent in Spain (P less than 0.0005) than in the other countries.

More than 60 per cent of the Spaniards studied are living near the north western coast of Spain. When the frequency of Pi^S among these were compared with that observed in the rest of the Spaniards, a value

TABLE 1

The Phenotypes Found in Some European and non-European Population Samples. The Expected Numbers Were Calculated from the Gene Frequencies in Norwegians (1)

		MM	MS	MZ	SS	FM	IM	SZ	MV	MW	Total	χ^2	
Spain	Observed	85	71	7	6	2	1	2	2	2	378	27[illegible] 7	n = 1
	Expected	315 29	16 4	11 93	0 3	9 51	0 86	0 27	0 29	0			P <0 000
Portugal	Observed	8	11	0	0	0	0	0	0	0	39	13 [illegible]	n = 1
	Expected	34 9	1 7	1 15	0 03	0 38	0 09	0 07	0 03	0			P <0 0005
Europe*	Observed	173	10	2	0	0	1	0	0	0	18[illegible]	[illegible]	n = 4
	Expected	1[illegible]	8 05	[illegible]53	0 1	4 68	0 42	0 13	0 14	0			0 0[illegible]<P <0 1
[illegible]an	Observed	23	3	0	0	0	0	0	0	0	[illegible]6	0 0002	n = 1
	Expected	23 27	1 13	0 77	0 01	0 65	0 06	0 02	0 0[illegible]	0			0 97[illegible]<P <0 99
Asia	Observed	68	0	0	0	0	0	0	0	0	68	7 8	n = 1
	Expected	60 8	2 96	[illegible]	0 01	1 71	0 15	0 05	0 0	0			P = 0 00[illegible]

*Except Norway Spain and Portugal

TABLE 2

Pi Gene Frequencies in Some European and non European Population Samples

		Pi^M	Pi^S	Pi^Z	Pi^F	Pi^I	Pi^X	Pi^W	No of subjects
Spain	No of genes	655	85	9	2	1	2	2	378
	Gene frequency	0 8664	0 1124	0 0119	0 0026	0 0013	0 0026	0 0026	
Portugal	No of genes	67	11						39
	Gene frequency	0 8590	0 1410						
Europe	No of genes	359	10			1			186
	Gene frequency	0 9650	0 0269			0 0027			
Latin america	No of genes	49	3						26
	Gene frequency	0 9423	0 0577						
Asia	No of genes	136							68
	Gene frequency	1 0000							
Norway	Gene frequency	0 9460	0 023	0 0157	0 0133	0 0012	0 0004		2830

Except Norway Spain and Portugal

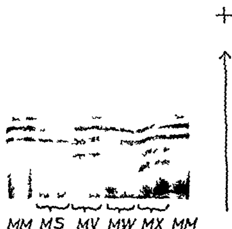


Fig 1
Photograph of part of an amido black stained starch gel showing the Pi phenotypes MM MS MV MW and MX

$0.6 < P < 0.7$ was obtained which indicates that this material did not provide any evidence that Pi^S is unevenly distributed in Spain in contrast to findings concerning the ABO blood groups (7)

The 68 subjects from Asia were all of phenotype MM while six or seven heterozygotes would be expected among a similar number of Norwegians This difference in distribution of Pi phenotypes is significant $P = 0.005$

The gene frequencies in the different population samples calculated by direct counting of genes are given in Table 2 By way of comparison the gene frequencies in Norwegians are listed below

According to Table 1 two cases of a new phenotype called MW were observed among the Spaniards This phenotype may be compared with the MV phenotype since both consist of four major zones of which the two in front correspond to the MM ones except that they possess about half their staining intensity The two major zones behind in the MW phenotype are somewhat slower and weaker than the V zones as judged from the MV phenotype Fig 1 is a photograph of an amido black stained starch gel where the phenotypes MV and MW are compared Fig 2 is a photograph of four antigen antibody crossed electrophoresis gels showing the relative protein concentrations of the different Pi zones in the phenotypes MM MS MV and MW It appears from both photographs that the two slow major zones in the MW phenotype are slower and weaker than the corresponding zones in the MV phenotype In the MV phenotype in Fig 2 M zone no 6 and V zone no 4 overlap and produce a very high peak while the corresponding peak in the MW phenotype is barely as high as the peak in front (M zone no 4

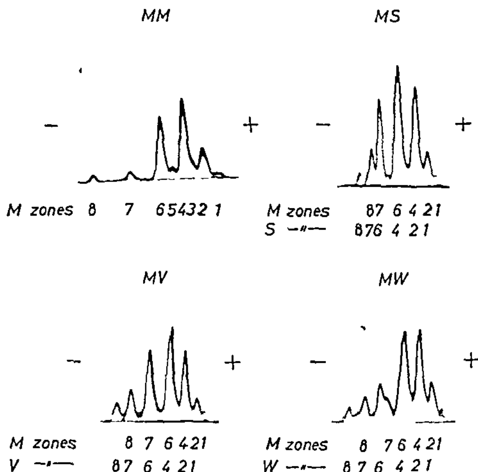


Fig 2

Photograph of antigen antibody crossed electrophoretic patterns of Pi phenotypes MM MS MV and MW The first electrophoresis was performed in acidic starch gel with the anode to the right as shown the second electrophoresis was performed in agarose gel containing anti alpha 1 antitrypsin from rabbit

+ W zone no 2) Similarly V zone no 6 and M zone no 7 overlap and produce a high peak while W zone no 6 and M zone no 7 are so far apart that they produce a double peak showing directly the relative protein concentrations

The MM pattern shown in Fig 2 consists of 8 protein peaks although peak no 3 is seen only as a shoulder to the right on peak no 4 In phenotype SS peak no 3 is clearly demarkated from ... 4 thus there is reason to believe that the allele product gel electrophoresis is a pattern of 8 protein zones (8) phenotypes several peaks consist of two or more and peaks no 3 and 5 can seldom be seen In F low each peak indicate the main constituent zo

The observations suggest that the MW phenotype is a new heterozygous phenotype resulting from the combination of Pi^M and a new allele for which the term Pi^W is proposed. Family studies will be needed to provide the definite proof of the correctness of this theory.

DISCUSSION

Unfortunately this material includes too few subjects from the individual Mediterranean countries to allow any comparison between the Pi gene frequencies in these and in the Iberian populations. Since samples obtained from Spaniards differ from the population sample of non-Iberian Mediterranean subjects there is reason to believe that Pi typing of a larger number of subjects from each country might give interesting information concerning the interrelations between populations in the Mediterranean area.

Because of the high frequency of Pi^S the Iberian populations should be especially well suited for studies of the possible linkage between the Pi system and other genetic markers.

Since it has been shown that the Pi phenotype ZZ is associated with a high risk of severe pulmonary disease it is possible that also other rare homozygous types, e.g. SS, might have associations to disease. Even in such investigations the Iberian populations would provide the best material.

There is no reason to believe that all the alleles within the Pi system have been detected yet. It is quite possible that studies of large populations, e.g. those of African origin, might reveal new alleles. In addition it should be remembered that the electrophoretic technique may not detect mutant types in which the net charge on the protein molecule has not been altered. Such variants may be detected if the mutation causes a significant change in the molecular weight or in the protein concentration of the allele product in serum when compared with the MM phenotype.

SUMMARY

Serum Pi phenotypes were studied in 655 foreign seamen on Norwegian ships and in 42 Tibetan refugee boys. The distribution of Pi phenotypes in population samples from Spain, Portugal and Asia differed significantly from that in Norwegians. The frequency of Pi^S was 0.1124 and 0.1410 among Spaniards and Portuguese respectively, as compared with 0.023 in Norwegians. The frequency of Pi^F was significantly lower among Spaniards than among Norwegians (0.0026 and 0.0133 respectively). In 68 subjects from Asia only phenotype MM was observed. A new phenotype called MW was observed in two Spaniards. This is probably a heterozygous phenotype resulting from the combination of Pi^M and a new allele for which the term Pi^W is proposed.

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